University of Alberta

Symmetry of Mouse Corpus Callosum Development

by

Christopher Dean

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Psychology

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Abstract

The physiological origins of cerebral asymmetry are largely unknown. Several theories have been forwarded, including one hemisphere maturing and forming contralateral callosal projections before the other and the existence of an organismic asymmetry gradient. Corpus callosum asymmetry was addressed by tracing axon growth at various stages of development in B6D2F$_2$ and BALB/cWah1 mice fetuses using the lipophilic dyes DiI and DiA. Morphologic prenatal asymmetry was studied in the developing paw using B6D2F$_2$ and BALB/cWah1 fetuses, and eye using B6D2F$_2$, C57BL/6J and DBA/2J fetuses. No population-level directional asymmetry was apparent in the growth of the callosum, eye or paw. However, a high degree of individual asymmetry was evident in all the systems studied. No evidence was found of an organism-wide asymmetry gradient; since there was no consistent pattern of asymmetry favouring one side within an individual. Instead, within each system the pattern of development was more consistent with fluctuating asymmetry. Using the common measurement of body weight, the amount of asymmetry in the external morphology was compared to the brain and found to be approximately equal. Finally, no significant strain differences in amount of asymmetry were evident, because the inbred BALB/cWah1, C57BL/6J and DBA/2J fetuses did not consistently exhibit larger amounts of asymmetry than the hybrid B6D2F$_2$ fetuses.

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List of Abbreviations

ACC - Corpus callosum

AMD - Average maximum distance

AP - Anterior to posterior

CC- Corpus callosum

CNS - Central nervous system

CPMB - Crossing point to main bundle

DA - Directional asymmetry

E - Embryological day

EBW - Estimated body weight

EBWDIF - Estimated body weight difference

FA - Fluctuating asymmetry

FC - Frontal cortex

GBG - Geschwind, Behan and Galaburda model

ISMB - Injection site to main bundle

JAVA - Jandel Video Analysis Software

ln - Natural logarithm

LR - Left or right

MB - Main bundle

MD - Minimum or maximum distance

ML - Midline

OC - Occipital cortex

PC - Parietal cortex

PFA- Paraformaldehyde

PFC - Prefrontal cortex

PT - Planum temporale

PTr - Planum temporale par triangularis

R-L - Right minus left

REA - Right ear advantage

TC - Temporal cortex

Tfm - Testicular feminization mutation

WT - Weight

Symmetry Of Mouse Corpus Callosum Development.

In 1861 Broca discovered the pivotal role of the left hemisphere in language function. Since that time the existence of asymmetries in the human brain have become well established and include morphological, neurochemical and functional differences, most notably with respect to left hemisphere dominance for language production and dextral motor processes (McManus & Bryden, 1992). The demonstration of functional hemispheric asymmetries has stimulated research into cerebral lateralization in both human and infrahuman species. Because language was long considered a uniquely human trait, the idea of neural lateralization in animals was controversial. However, evidence accumulated over the last two decades supports the existence of cerebral lateralization, both structurally and functionally, in a range of species (Fitch, Brown, O'Conner & Tallal, 1993).

In general, there are three types of asymmetry found in the developing organism. The most obvious is directional asymmetry (DA), a consistent bias toward one side of the body within a species (eg. human handedness) resulting in a skewed distribution of right minus left (R-L) differences centred around a mean significantly different than zero (Markow, 1992). Fluctuating asymmetry (FA) occurs when the usual state of a bilateral trait is symmetric, but there is a tendency for one side to be larger than another, giving R-L differences with a normal distribution around a mean of zero (Phelan & Austad, 1994). Finally, antisymmetry (AS) is the result of pronounced differences between the left and right sides of the body, but occurring with equal frequency on each side (eg. paired claws in the lobster; Govind, 1992). This gives a bimodal distribution of

differences around a mean of zero (Palmer & Strobeck, 1986).

The aforementioned lateralities may be classified, in both human and subhuman populations, as either individual or populational. Paw preference in mice is individual because each mouse exhibits a distinct left or right bias but the population as a whole shows no side preference (Collins, 1985), whereas human hand preference is a populational asymmetry because individual preferences exist and the population is also biased in one direction. In general, laterality among animals tends to be less populational than individual.

Within the present paper the differing forms, interrelations and possible origins of asymmetry will be discussed. First, the relationship between anatomic asymmetry and laterality is presented. The focus will be upon handedness and language, in addition to addressing the possibility that these characteristics may be sexually dimorphic. For each of these topics the evidence from both human and non-human research will be considered. Next the developmental aspects of asymmetry will be examined, with specific attention paid to the role of the corpus callosum.

Anatomic Asymmetry

Some of the most dependable and consistent directional asymmetries in humans are anatomic. The left hemisphere exhibits a longer Sylvian fissure, larger plenum temporale and a greater volume of the globus pallidus (Galaburda, Sanides & Geschwind, 1978; Kooistra & Heilman, 1988). These and other morphological differences have been touted as possible substrates of the well established functional lateralization seen in the human brain, though the hypothesized links between structure and function are equivocal

(Witelson & Kigar, 1988). Cerebral control of a broad spectrum of autonomic physiological activity is also lateralized within the brain, both sides of the brain playing different roles regulating these processes. Physiologic functions that are regulated by the right hemisphere in right-handed individuals include cardiac activity, blood pressure, cortisol reactivity and immune-related activity (Wittling & Schweiger, 1993).

Relatively few rightward asymmetries have been found in the human brain that may correspond to functions that have been proposed to be lateralized to the right hemisphere (e.g. visuospatial abilities). However, right hemisphere asymmetry has been postulated to be associated with spatial giftedness and musical talent (Schlaug, Jäncke, Huang & Steinmetz, 1995). Additionally, Jäncke, Schlaug, Huang, and Steinmetz (1994) have suggested that part of the right inferior parietal lobule (designated the planum parietale) may be a central point subserving the function of directed attention.

Asymmetry in cortical anatomy is well established in the animal kingdom. The Sylvian fissure and lateral geniculate asymmetries seen in humans have also been found in chimpanzees and some great apes (Yeni-Komshian & Benson, 1976; Hopkins & Bard, 1995). However, similar results have not been obtained in studies of monkeys where asymmetries, if they exist, may be more subtle (Heilbroner & Holloway, 1988). Reliable asymmetries can be found in the habenular nuclei of reptiles and amphibians and the tracheosyringeal portion of the hypoglossal nucleus in thalamofugal visual projections of avians, respectively (Rajendra & Rogers, 1993). There is also evidence for morphological asymmetries in the rodent brain; for instance, a variety of asymmetries has been discovered in the neocortex and hippocampus of rats and mice. Reid and Juraska (1992a)

examined the thickness of specific cortical layers at several locations in the cerebral cortex of littermate Long-Evans rats. The authors concluded that although asymmetries were found at several locations, the direction of asymmetry was somewhat inconsistent. Additionally, in male rats the thickness of layers II-III, V and VI was greater in the primary motor, sensorimotor and visual cortices. Diamond, Young, Singh and Johnson (1983) have also reliably shown that the right neocortex in male Long-Evans rats is thicker than the left. Ward and Collins (1985) reanalyzed the mouse cerebral asymmetry data of Kolb, Sutherland, Nonneman and Whishaw (1982) and confirmed that a rightward gross cerebral asymmetry exists also in mice. Cassells, Collins and Wahlsten (1990) also found that the strongly lateralized HI strain of mice have larger brains than the more weakly lateralized LO mice. This difference in brain size was positively related to the difference in CC size and was posited to play a role in the differing degree of lateralization between the two strains. Anatomical asymmetries in cats, dogs, rabbits, rats and mice are similar, with the right hemisphere being larger and heavier than the left (Lent & Schmidt, 1992).

Functional Asymmetry

In the human population the most salient examples of lateralization are language and handedness (Calnan & Richardson, 1976; Larsen, Skinhoj & Lassen, 1978). Handedness is most commonly defined as the tendency to use either the right or left hand preferentially in the completion of manual tasks. Among humans being right-handed, or dextral, is most common; however, approximately 10% of humans show the opposite preference and are left-handed or sinistral. In addition, a small sex difference exists, with

a slightly larger portion of males being left-handed. The origins of both hand preference and sex difference remain ambiguous, but a variety of theories have been forwarded. These hypotheses include genetic causes (Bishop, 1990), social causes (Porac & Coren, 1981), atypical brain development (Geschwind & Galaburda, 1985) and pathological left-handedness (Satz, 1972).

The genetic component of handedness has been the subject of intense study; however, the mechanisms thought to be responsible have not been well elucidated. One genetic model proposed by Levy and Nagylaki (1972) posited that handedness and cerebral dominance are each controlled by an independent locus, with both loci being diallelic. In this model the majority of the population would be homozygous dominant or heterozygous for left cerebral dominance (LL, Ll) and for handedness contralateral to the dominant hemisphere (CC or Cc). Annet (1994) has forwarded a one-gene model of handedness. In this model there are two alleles possible at a single right shift locus and being dextral is the result of receiving a dominant allele (*rs*++, *rs*+-) while sinistrality is a random consequence of recessive homozygosity (*rs*--). In general, genetic models assume that the genomic variation necessary for handedness is maintained through heterosis (McManus & Bryden, 1992). However, no conclusive evidence exists that demonstrates handedness is exclusively a genetic phenomenon. Coren and Halpern (1991) after reviewing 13 twin studies concluded that there was little difference between monozygotic and dizygotic twins in terms of their concordance to be right- or left-handed. Furthermore, family studies have generally found that within a family it is the strength rather than the direction of handedness that appears to be inherited (Coren &

Porac, 1980). Nevertheless, there are factors that point out biology plays a role at some level. For instance, Hepper, Shahidullah and White (1991) used ultrasound to study the thumb-sucking preference of fetuses and found a bias for sucking the right thumb that was maintained throughout the pregnancy. The authors interpreted this finding as evidence for a specific hand preference being established prior to birth. Additionally, the base rate of sinistrality has remained relatively consistent over time despite being selected against culturally.

There is some evidence that the preponderance of dextrals in the population may in part be due to cultural biases that favour right-handers. Hand preference does not become fixed until after 3 years of age (Bishop, 1990), so there is ample time for parental influences to be exerted. For example, infants may imitate the motor actions of their parents or the parent may make explicit suggestions regarding appropriate hand use (Masur, 1988). However, cultural explanations for handedness have difficulty explaining how dextrality became common across all human societies. As discussed above, the cultural bias against left-handed individuals has been intense, and if cultural factors were the primary cause of handedness then sinistrality should be extremely rare. Also, there have been extremely few societies documented where handedness was either normally distributed or skewed differently from the current population (Corballis, 1983).

The existence of left-hand preference has been postulated to be associated with pathology, i.e. brain injury or developmental disorder. As such, sinistrality has been found to be more prevalent amongst clinical groups, including mental retardation (McManus, 1983), learning difficulty (Bishop, 1990) and autism (Gillberg & Gillberg,

1983). However, in contrast to these findings, left handed individuals are also more likely to be found amongst those who excel academically or are musically gifted (Temple, 1990; Schlaug et al., 1995). It is also possible that peri- or prenatal stressors may be associated with the aforementioned physical or neurological problems and that sinistrality is a marker for this relationship. In a meta-analysis of the relationship between birth stress (e.g. prolonged labour, birth-related anoxias) and left-handedness Searleman, Porac and Coren (1989) reported a significant relationship linking stress factors to increased levels of sinistrality. Similarly, Coren (1994) proposed that twinning may also be a prenatal stressor and in concert with this hypothesis multiple births were associated with an increased incidence of both left-handedness and inverted writing posture. However, when O'Callaghan, Burn, Mohay, Rogers and Tudehope (1993) examined high risk infants they found that low birth weight was associated with a heightened level of left-handedness, but support for the pathological mechanisms (e.g. apneas requiring ventilation, symptomatic hypoglycemia) leading to left-handedness was found primarily in infants of normal birth weight. The authors suggested that mechanisms other than brain damage may lead to left-handedness in premature infants. Nevertheless, birth stress may provide an alternative explanation to the genetic models because this model can account for the findings of most family studies. That is, the female child of a left-handed mother may be sinistral due to perinatal stress factors. These stress factors may also be exhibited by the daughter when she gives birth to children, and in this way subsequent children may have a preponderance for left-handedness. A possible physiological mechanism is that the stressor affects the normal neuronal migration patterns and this in

turn leads to an atypical connectivity, which manifests itself as a deviation from the normal pattern of dextrality.

The manipulative skills discussed above are not unique to humans, but in contrast with the human data, results from studies of infra-human species have indicated that even though individuals exhibit hand and foot preferences, there is no consistent bias in the population (Bradshaw, 1991). Within the animal kingdom rodents and primates have all been extensively studied using reaching tasks. In mice laterality tasks have received considerable study since the landmark work by Collins (1968; 1969) who attempted to select lines that showed a paw preference. While this result did not materialize, Collins (1985) was able to demonstrate selection for degree of preference rather than direction. This paw preference was found to be stable over long periods of time and was not task specific. Further work has generally supported the contention that the genetic aspect of paw preference in mice is related primarily to degree rather than direction (Betancur, Neveu & LeMoal, 1991; Signore, Chaoui, Nosten-Bertrand, Perez-Diaz & Marchaland, 1991). Biddle, Coffaro, Ziehr and Eales (1993) conducted reciprocal F_1 crosses on several strains of mice that differed in their degree of lateralization. From the results obtained the authors posited that degree of laterality may be an additive genetic trait but that neither maternal nor X-linked effects were apparent.

The paw preference work done with mice has also found evidence of both a sex difference and a cultural aspect to handedness. Collins (1968) originally reported a sex difference with females showing a higher degree of lateralization, and support for this finding has been provided by Signore, Nosten-Bertrand et al. (1991). However, Waters

and Denenberg (1994) after analyzing paw preference in a large group of hybrid mice failed to find any sex difference, though these investigators used a different reaching task. Support for the cultural aspect of handedness in mice was provided by the work of Collins (1975). Collins used a sample of equally left- and right-pawed mice and exposed them to an environment conducive to right-paw use and found that the majority of mice began feeding with their right paw preferentially. Further, Collins (1988) used a sample of B6D2F_1 mice in an experiment that required the animals to swing a pendulum door to the left or right to obtain food. Half the mice were allowed to observe another animal complete the task prior to being tested. The results from this study are twofold. The test subjects completed the task more quickly than the control animals that were placed behind a visual barrier; and those animals that had observed a "teacher" also exhibited the same paw preference as the "teacher" mouse. Interestingly, the latter effect was evident only in the male mice. These results point out the possibility that observational learning may have a role in establishing paw preference.

Among right-handed humans the phenomenon of left-hemisphere language dominance has been demonstrated using a number of different experimental paradigms, including dichotic listening, regional cerebral blood flow, evoked potentials and positron emission tomography (Rosen, Galaburda & Sherman, 1990). In contrast, although some left-handers do exhibit left-hemisphere language dominance, this characteristic is not as universal because many also have language represented bilaterally or in the right hemisphere (Kimura, 1983). Evidence of this anomalous dominance in sinistral individuals has been supported by stroke patients (Bryden, Hecaen & DeAgostini, 1983),

hemispheric anesthesia studies (Ratcliff, Dila, Taylor & Milner, 1980) and behavioral studies (McBride, Cherry, Kee & Neale, 1995).

The planum temporale is the area of the supratemporal plane behind the primary auditory cortex, identified as Wernicke's area, and long considered a cortical area essential to language (Geschwind & Levitsky, 1968). Planum temporale asymmetry has been consistently found by a number of different investigators using a variety of techniques (Pieniadz & Naeser, 1984; Steinmetz et al., 1989) and in embryos as young as 29 weeks gestation (Witelson & Pallie, 1973). Based on the commonality of these results, many researchers have postulated a causal relationship between the planum temporale asymmetry and language ability. That is, the planum would represent a physical substrate for left-hemisphere specialization and that asymmetrical development may set a pattern for cortical development for language function (Habib, Robichon, Levrier, Khalil & Salamon, 1995). However, some controversy still exists, due mainly to differences in defining the PT. Loftus et al. (1993) included the posterior ascending ramus in their PT area and failed to find the usual leftward asymmetry. Because a large extent of the peri-Sylvian area plays a role in language, investigators have also searched anterior regions for evidence of cortical asymmetry. Falzi, Perrone and Vignolo (1982) measured the depth of the convolutions in the triangular and opercular regions of the inferior frontal gyrus. These investigators found a significant leftward asymmetry in the sample of 12 right-handed subjects. Further, Foundas, Leonard and Heilman (1995) used thin sagittal MRI scans of the PT and pars triangularis (PTr) to ascertain the extent of asymmetry in the frontal operculum. Their results confirmed the leftward asymmetry of the PT and

suggested that the PTr also has an asymmetry in that direction amongst the right-handed subjects.

There has been a long-held belief that the language capabilities of females are represented more bilaterally than in males (Rugg, 1995). Most evidence for this position comes from lesion studies because unilateral left hemisphere lesions tend to result in a higher incidence of aphasia in males. In addition, males demonstrate greater differences between verbal and non-verbal intelligence after lesions to either hemisphere (McGlone, 1980). Shaywitz et al. (1995) have provided further evidence that language processes may be more lateralized in males, with females showing a greater bilateral representation. The authors conducted functional MRI scans of the inferior frontal cortex while presenting nonsense words and asked the subjects to decide whether they rhymed. Results from the MRI indicated that brain activation was more fully lateralized to the left inferior frontal gyrus in males, whereas in the female subjects the inferior frontal gyrus activity was occurring bilaterally. The authors posited this to be primary evidence for functional sex differences in phonological processing.

Lateralization with regards to what may be thought of as "language" has also been demonstrated in animals. Nottebohm and his colleagues determined that song production was lateralized to the left hypoglossal nucleus of the male zebra finch (Nottebohm, Stokes & Leonard, 1976; Nottebohm, 1977). Research with Japanese macaques has shown that they exhibit a right ear advantage (REA) for discriminating the coos of their own species. The REA seen in the macaques has been interpreted to represent a left hemisphere specialization for language processing. Evidence in favour of this position

includes lesion studies where damage to the left but not the right hemisphere impairs the macaque's ability to discriminate the coos (May, Moody & Stebbins, 1989). There has also been evidence for left-hemisphere advantage of acoustic processing in rodents. Female house mice showed a lateralized advantage for discriminating bursts of noise from ultrasonic pup calls (Ehret, 1987). However, Fitch et al. (1993) demonstrated a REA for auditory temporal processing in male rats but not in handled female rats. This evidence parallels some of the findings with human subjects where the REA is consistently found but often with a great deal of variability present in the sample.

The differences between the sexes suggests that neuroendocrine factors may be developmentally important for cerebral lateralization. The finding of human sex differences in lateralization has promoted the view that early hormonal exposure may influence the development of lateralization mechanisms and subsequent hemispheric specialization. One of the most widely cited explanations for this finding has been that of Geschwind, Behan and Galaburda (GBG) (Geschwind & Behan, 1982;Geschwind & Galaburda, 1985) who advanced an influential model of asymmetry predicated upon the causal effects of exposure to prenatal testosterone. One of the primary supports for this model is the evidence that gonadal steroids exert a great deal of influence on the development of sexual dimorphism in the nervous system (Toran-Allerand, 1986).

Further evidence for neuroendocrine influences upon development of laterality comes from animal research. Nosten, Roubertoux, Degrelle and Leboyer (1989) tested the strength of laterality in mice with a testicular feminization (*Tfm)* mutation that causes testosterone insensitivity. The authors posited that because of the decreased influence of

testosterone the male *Tfm* mice and their female sibs would be more strongly lateralized than the males without the mutation. This was the result obtained and the authors proposed that the effect of increased testosterone sensitivity was to diminish hemispheric asymmetry thereby inducing ambilaterality. Diamond et al. (1983) have also demonstrated a sex difference in cortical structure, with the males exhibiting a more pronounced asymmetry.

However, Bryden, McManus and Bulman-Fleming (1994) have recently called into question the role of prenatal testosterone in developing neuroanatomical and functional asymmetries. By reviewing the literature relating to the GBG model the authors concluded that beyond the greater incidence of language disorders among men (McManus & Bryden, 1992) little empirical support exists for the causative effect of testosterone upon developing laterality.

The reduced explanatory power of the GBG model has led to investigations of other environmental perturbations and neurochemical alterations that may result in behavioral laterality. Stress may play a role in both structural cerebral asymmetry and behavioral lateralization because stressors have direct effects on the catecholaminergic system, as well as the hormones of the pituitary-adrenal axis and other immunological factors (Anisman & Zacharko, 1990). Neonatal handling has been shown to enhance functional lateralization in adult male rats as measured by unilateral lesions (Denenberg & Yutzey,1985). Exposure to stressors, such as handling, activates dopamine neural systems in the brain, including prefrontal cortex (PFC), nucleus accumbens and striatum. Carlson, Fitzgerald, Keller and Glick (1993) exposed animals to both chronic and acute

stressors and found that uncontrollable stressors caused an increase in the DA/DOPAC ratio specific to the right PFC. The authors suggested that the right side of the PFC may be differentially sensitive in responding to stress, as seen with elevated DA/metabolite ratios. Additionally, within some strains of inbred mice the strength of laterality has been correlated with immune functioning. Left-pawed females of the C3H/He strain exhibited increased lymphocyte proliferation after introduction of an antigen (Neveu, Barneoud, Vitiello, Betancur & LeMoal, 1988). Similarly, endogenous natural killer cell activity was higher in right-pawed than left-pawed male C3H/OuJIco mice (Betancur, Neveu, & LeMoal, 1991). Neurochemical hemispheric asymmetries in the human brain discovered with post-mortem biochemical analyses include glutamic acid decarboxylase, gamma aminobutyric acid, choline acetyl transferase and dopamine in the basal ganglia (Glick, Ross & Hough, 1982). These asymmetries could underpin some of the functional asymmetries seen earlier, but the link is unproven and the causal direction has not been elucidated.

The aforementioned relationships between functional lateralization and cortical asymmetry provide some valuable and attractive correlations. However, a causal link has not been elucidated and the direction of causation, if one exists, remains obscure. Greenough, Larson and Withers (1985) and Greenough and Anderson (1991) have provided reports of functional and morphological modifications induced by lateralized reaching tasks. Further, Diaz, Pinto-Hamuy and Fernandez (1994) trained early post-weaning male rats in a lateralized reaching task. The results indicated that all the experimental animals developed structural modifications in the hemisphere contralateral

to the reaching forelimb. The authors suggested that the majority of this restructuring reflects an increase in dendritic arborization and that it is possible to induce brain asymmetries in restricted cortical regions. A linear relationship between structural and behavioral asymmetry has been posited by Bernasconi-Guastalla, Wolfer and Lipp (1994) studying mossy fibre projections and water maze navigation in mice. The authors found a consistent relation between mossy fibre projection asymmetry and turning preference that may suggest a functional relationship.

Asymmetry and the Corpus Callosum

Another approach to linking anatomical and functional asymmetry is through the corpus callosum (CC). The CC is the primary fibre bundle to provide the communication between hemispheres and is pivotal for hemispheric integration (Sperry, 1982). As such, the CC is often considered crucial for the expression of hemispheric specialization and lateralization (Habib et al., 1995). Denenberg (1981) has postulated that the CC affects the development of lateralization by allowing a specific area within one hemisphere to exert an inhibitory action upon its homologue in the corresponding hemisphere, thereby creating a functional asymmetry. If this were the case then a defective CC would result in a decrease in lateralization due to independent hemisphere development. Alternatively, the CC may facilitate the development of a pre-existing functional asymmetry as a consequence of increased inter-hemispheric orchestration. In this case, the result of an aberrant or absent CC would be an increased level of lateralization.

Difficulty remains in linking structural aspects of the CC with observable functional characteristics. For example, Witelson (1985; 1989) provided some evidence

that the midsagittal area of the callosum, specifically the callosal isthmus, is larger in subjects that are not consistent right-handers. It was hypothesized from these results that this inequality may be a physiological substrate for the more bilateral representation of cognitive function seen with non-righthanders. The increased amount of connectivity between hemispheres may lead to reduced interhemispheric transfer time. This hypothesis is based on the work done with dichotic monitoring, in which right-handers have exhibited a distinct right-ear advantage, presumably due to the lateralization of language function to the left hemisphere. The decreased efficiency of dichotic stimuli presented to the left ear is thought to be the "transfer time" required for the auditory message to travel from the non-specialized right hemisphere across the CC to the left (Geffen & Caudry, 1981). It is upon results like these that the "callosal relay model" has been developed. Corpus callosum size is thought to be inversely related to degree of asymmetry (Rosen, Galaburda & Sherman, 1990); those subjects with reduced reaction times should presumably have less lateralized brains and hence a larger CC. This was the hypothesis that Jäncke and Steinmetz (1994) tested, only to find that neither the CC nor the size of any CC subarea correlated with the auditory or motor reaction time differences. The authors suggested that their results were supportive of the "direct access model". In this model both hemispheres are capable of cognitive functions (e.g. language) but one hemisphere is more efficient. In this case the differences in reaction times are reflective not of callosal transfer time but processing time within a hemisphere.

Another area of investigation that may shed some light on the functional role of the CC is in acallosal or callosotomized subjects. The prevalence of corpus callosum

agenesis (ACC) in the general population is unknown because many cases go unnoticed, but agenesis provides an ideal framework to test the functional limitations of the CC. In the hospital population ACC is primarily found within the developmentally disabled (Jeret, Serur, Wisniewski & Lubin, 1987) and is commonly associated with mental retardation and seizures. However, these dysfunctions often arise as a consequence of general CNS malformation rather than the direct result of an absent CC. The degree of lateralization in ACC subjects will depend upon how intimately linked the CC is to development of hemispheric specialization. The CC may either facilitate a preexisting asymmetry or, through an inhibitory process, aid in the development of an asymmetry (Denenberg, 1981). The results from studies of language tasks in ACC subjects have been inconsistent. Geffen (1981) found language to be represented more bilaterally in a subject with a partial callosal section, but the majority of the results have found that lateralization persists in ACC individuals (Chiarello, 1980). Lassonde, Bryden and Demers (1990) compared acallosal, callosotomized and hemispherectomized subjects on dichotic listening tests. The results indicated that language function was more lateralized in the ACC subjects than matched controls and the authors cited this as possible evidence that functions may be asymmetric initially and that CC activity serves to reduce the level of asymmetry. Additionally, acallosals tend to exhibit distinct hand preferences (Sauerwein & Lassonde, 1994), an occurrence which would not be expected if hemispheric specialization was dependent upon an intact corpus callosum. Cognitive abilities are able to develop normally in acallosal subjects and when matched controls are used the only deficits that appear to separate normal and ACC subjects are sensorimotor

(Sauerwein & Lassonde, 1994). Cognitive studies have also been carried out extensively in commissurotomy patients. The work of Gazzaniga (1970) and Sperry (1968) established the credentials of hemispheric specialization and confirmed the importance of the CC for inter-hemispheric communication. The level of cognitive functioning does not appear to be reduced in patients who have had their CC sectioned and similar to the ACC patients, subjects with callosotomy show some motor function deficits (Jeeves, Silver & Jacobsen, 1988).

Within the animal population four inbred mice strains have been found that exhibit agenesis of the CC to some degree: BALB/c (including substrains BALB/cWah1, BALB/cWah2, BALB/cCF), I/LnJ, 129/J and ddN (Wahlsten & Ozaki, 1994). Mice of the I/LnJ strain never develop a CC, while in the remaining strains the CC may be normal, small or entirely absent. Acallosal strains of mice allow investigators to determine if a relationship exists between CC integrity and anomalous lateralization (Lipp & Wahlsten, 1992); for instance, the relationship that may exist between laterality of paw preference and the extent of callosal connectivity. Ward, Tremblay and Lassonde (1987) tested the degree of ambilaterality in BALB/cCF and 129/J mice and found that the size of the CC was significantly correlated with the degree of paw preference, but only in the 129/J mice. However, Schmidt, Manhães and de Moraes (1991) studying the direction and magnitude of paw preference found BALB/cCF mice to be more strongly lateralized than hybrid controls. The authors also reported a tendency for left-paw preference within the BALB/cCF mice, especially in mice with an abnormal corpus callosum. Further, Gruber, Waanders, Collins, Wolfer and Lipp (1991) tested I/LnJ,

C57BL/6J and DBA/2JZur mice in a lateralized food reaching task. The authors found the acallosal mice to have a reduced degree of paw preference, the degree of paw preference increases as the animals age, and the increased laterality was more prevalent in females. The authors concluded that CC development was an important factor in determining the degree of paw lateralization. In contrast, Biddle et al. (1993) found the SWV mouse strain, which does not exhibit any anomaly of the CC, to also be weakly lateralized. Laroche and Ward (1992) tested the reflex responses of BALB/cCF mice for three postnatal weeks. The authors concluded that an intact CC was not mandatory for normal maturation of sensorimotor cortex because the proportion of animals showing asymmetric responses decreased at an equal rate between normal and abnormal mice. Similarly, Bulman-Fleming, Wainwright and Collins (1992) studied the effects of handling on CC size and paw preference in BALB/c mice. The authors concluded that the degree of paw preference was not significantly correlated with either CC agenesis or CC area.

de Lacoste-Utamsing and Holloway (1982) and de Lacoste, Holloway and Woodward (1986) have been vocal supporters of anatomic sex differences in the CC, having reported that overall area, and more specifically the splenium, is larger in human females. The authors proposed this anatomic difference as the basis for alleged functional differences in the cognitive performance of males and females. In contrast to these results other investigators have either failed to find these relationships (Oppenheim, Lee, Nass & Gazzaniga, 1987; Byne, Bleier & Houston, 1988) or have interpreted their results differently. For example, Kimura (1983) proposed that the sex differences were the result

of different organizational language constructs between the sexes, but that these differences were occurring in an equally lateralized left hemisphere. Bishop and Wahlsten (1995) conducted a meta-analysis on data relating to human CC morphology and sex differences. The authors found that males tended to have larger corpora callosa, but when the effect of brain size was partialed out the differences in the CC between males and females were negligible. Furthermore, the authors pointed out that the only result obtained by de Lacoste-Utamsing and Holloway (1982) that reached statistical significance was splenial width. This finding is unlikely to contribute to a cognitive sex difference because splenial width corresponds to the shape of the CC rather than connectivity and any potential differences would be found between the occipital cortices rather than association cortices.

The investigations of possible sex differences in human CC morphology have led to a number of similar investigations in other species. Juraska and Kopcik (1988) and Juraska (1991) have investigated the possibility that the rat CC may be sexually dimorphic, but the results have been equivocal. Juraska and Kopcik (1988) raised male and female Long-Evans rats in either complex environments or in isolation. The midsagittal area of the CC was measured in thirds, but no sex differences were apparent, although the authors did find that female rats had 7-10% more unmyelinated axons in the splenium. Instead, the posterior third of the CC differed significantly between the rats raised in the complex and isolated conditions. In a follow-up to the difference in splenial axon number Kopcik, Seymour, Schneider, Kim-Hong and Juraska (1992) used horseradish peroxidase to examine the distribution of callosal projection neurons from

the visual cortex; but the authors failed to find any sex difference in the distribution of projecting neurons.

Juraska (1991) points out that simply analyzing the overall size or shape of the CC is not sufficient in deciding what characteristic of CC morphology is most relevant to behavior. Because of the malleability of the CC to differential environments, experiental variables may either masquerade as sex differences, if small samples are used, or obscure subtle sex differences. Further, the most basic assumption that the size of the CC has a linear relationship with the number of axons may not be accurate. Lamantia and Rakic (1990) studied adult Rhesus monkeys and concluded that no relationship existed between the number of axons and the size of the corpus callosum. Instead the size of the callosum may be a function of the number, density and diameter of the axons, in addition to the amount of myelin. In general, it is the sensory areas that are connected by large diameter heavily myelinated fibres, whereas the higher-order processing areas are represented by smaller diameter less-myelinated axons (Aboitiz, Scheibel, Fisher & Zaidel, 1992) which would not facilitate rapid communication. Investigating these issues is predicated on the assumption that there exists distinct interhemispheric communication pathways and that some of these pathways are likely to be involved with lateralized functions. Instead, an alternative explanation may be that differences in callosal size and shape may occur independently of callosal function (Juraska, 1991). That is, lateralization and CC shape may be under the control of similar developmental factors but no causal relationship exists between them.

Mechanisms and Origins of Asymmetry

At one time the brain was thought to be symmetrical at birth and as development proceeded postnatal influences determined direction and possibly the magnitude of asymmetry (Rosen et al., 1990). However, more recent evidence from work done in the area of speech perception has done much to refute this assumption. Studies using the high-amplitude sucking paradigm in conjunction with dichotic listening have found that infants showed a REA for a speech stimulus and could also discriminate and dishabituate to stimuli, further supporting the idea that children may be lateralized from birth (Entus, 1977; Eimas, 1985). Because the CC is one of the most important connections between the cerebral hemispheres, the possibility exists that one of the mechanisms of cerebral asymmetry may act through the callosum. For example, one hemisphere may send more axons across the CC, thereby establishing a pattern of dominance early in development (Weiskrantz, 1977). Witelson and Nowakowski (1991) hypothesized that one of the determining factors in establishing patterns of functional asymmetries is the naturally occurring amount of callosal axon loss during fetal and early postnatal development. In this model greater right hemisphere axon loss over time leads to the patterns of right handedness seen in the population. The other possibility is that the brain is already asymmetric and uneven cell loss furthers a preexisting asymmetry or maintains an existing one. Lassonde et al. (1990) proposed that anatomical asymmetries may appear first and partially determine the amount of future callosal fibre retraction. In this proposal the CC has a role similar to that assigned it by Schmidt (1994), i.e. the CC may modulate rather than create asymmetries by allowing an incipient lateralization to develop so that the hemisphere with fewer callosal connections will become more specialized in its

functioning. Rosen et al. (1990), measuring the total architectonic volume of the somatosensory and somatomotor cortices, established that there were more callosal terminations in the symmetrical than asymmetrical brain regions. These conclusions buttressed their hypothesis that in asymmetrical brains, the smaller regions have fewer cells and subsequently a decreased number of callosal fibres, suggesting asymmetric interhemispheric connections. Rosen et al. (1993) investigated which type of cells may contribute to hemispheric asymmetry by selective staining for parvalbumin-specific long projecting neurons and vasoactive intestinal peptide non-pyramidal neurons. The authors concluded that the concentration of parvalbumin-staining neurons increased only in the larger side, possibly indicating qualitative differences in the cellular content of the larger hemisphere. This conclusion further supports previous findings that alterations of cytoarchitectonic intrahemispheric borders and differences in architectonic volume were attributable to differences in cell number (Rakic & Williams, 1986; Reid & Juraska, 1992b). Nevertheless, despite the success of studies in demonstrating asymmetric cellular content between hemispheres the familiar problem of causal direction has yet to be elucidated.

The evidence accumulated thus far would indicate that cell-cell signaling is clearly involved in the regulation of right-left development but the complex intercellular interactions are a little known facet of asymmetrical development. Since most vertebrates have consistent right-left differences, the question arises of how these may be represented in the embryonic axes of a population (Yost, 1995). To generate a population-level side difference requires not only a mechanism for determining the asymmetry but a

nonrandom process whereby this asymmetry is maintained (Klar, 1994). Brown and Wolpert (1990) suggested that the development of asymmetries involves three separate processes. The authors postulated that cells are polarized to create a midline and that a concentration gradient exists from right to left. Accordingly, this molecular asymmetry is then converted to the cellular level and interpreted by multi-cellular organs which develop preferentially on one side or the other. Central to this theory is a further mechanism that creates random right-left asymmetries at the cellular level and that is susceptible to a bias generated at the molecular level. In effect, this model presumes that asymmetries are generated subcellularly and in the event that this control is eliminated population asymmetries are not reversed; instead dominance of the right or left is a random event. Yost (1991) has provided evidence that in Xenopus an uneven allotment of intracellular components (e.g. microtubules) may establish an unequal dorsoventral axis in the first cell cycle. This type of intracellular gradient may provide the first step that leads to further cell-cell signalling as a possible cause of asymmetry. The Brown and Wolpert model posits random right-left asymmetries result when the normal gradient process breaks down. This possibility has been borne out in studies using the embryos of mice homozygous for the *iv* gene found on chromosome 12. The embryos are relatively undamaged but the internal organs are oriented randomly along the right-left axis (Seo, Brown, Ho & Anderson, 1992), that is, each organ ends up on one side or another independent of any adjacent organ. The lack of any pattern would lead to the conclusion that each organ's primordia receive the same asymmetric signal but interpret this signal independently (Yost, 1995).

Present Study

Significant biological asymmetries range from differentiation of sides in single cells to organ patterns across multicellular organisms. Despite extensive research little is known about how or why an organism becomes asymmetrically organized. Pansera (1994) suggested that cerebral asymmetry may allow the organization of neural circuits to be accomplished more efficiently. Nevertheless, the existence of this phenomenon within the brain is an area currently under intense scrutiny and subject to much media speculation (see Edmonton Journal (September, 1994), Newsweek (March, 1995), Time (January, 1992)). The experiments that follow will test a number of hypotheses and help to address some of the questions surrounding the relationship between asymmetry and the corpus callosum.

Hypothesis 1: Asymmetric CC development has been proposed as a mechanism of cerebral asymmetry (Witelson & Nowakowski, 1991). Therefore, using prenatal mice the present investigation addressed the question: does the mouse CC develop asymmetrically? Investigations into the extent and distribution of prenatal CC axons were accomplished using the lipophilic carbocyanine dyes DiI and DiA. Both forms of the dyes, crystal and solution, were injected into the brains of prenatal mice that had been intracardially perfused. These sensitive fluorescent dyes exhibited anterograde and retrograde labeling in long-distance CNS pathways by penetrating and diffusing along the lipid bilayer of the cell (Baker & Reese, 1994). As a result carbocyanine dyes have been an important development in the investigations of callosal axon origin and termination (Elberger, 1994) as well as growth cone morphology (Ozaki & Wahlsten,

1992).

Hypothesis 2: Within an organism there may exist an overall asymmetry gradient that directs the development of left- or right-side dominance (Brown & Wolpert, 1990). Therefore, the present study investigated the question: do external morphological characteristics exhibit developmental asymmetry? To this end the eye and the forelimbs were selected to study. Eye closing may be useful because the stages of eyelid development are common to all mammals (Harris & McLeod, 1982), and digit fusion is one of several temporary fusions taking place at this same time, including that of pinnae to the scalp (Maconnachie, 1979). One of the advantages of measuring the external morphology is that these sequences are sensitive to a variety of environmental and genetic perturbations (Tyndall & Sulik, 1991) and are therefore more likely to exhibit asymmetry. In contrast, asymmetry is thought to be reduced in traits that are more closely linked to the fitness of the organism (Clarke, 1993). Finally, genuine asymmetry in mouse neural development may be extremely subtle and studies of prenatal ontogeny may be aided by studying the external morphology of the fetus (Wahlsten, 1981).

Hypothesis 3: Genetically heterogeneous organisms should be healthier and exhibit more symmetrical development patterns than genetically homogeneous organisms (Wahlsten, 1981). The role of genetics is probed by using different strains of mice. Normal hybrid strains, genetically pure inbred strains and mice with preexisting CC defects were utilized so as to assess the potential differences in growth in a number of genetically distinct mice.

EXPERIMENT 1 - MULTIPLE DYE CRYSTALS

Introduction

This experiment was conducted to test the hypothesis that the arrival of CC axons at midline may be asymmetrical. To that end the lipophilic dye crystals DiI and DiA were used to trace the extent of development in prenatal mouse fetuses. Using the same methodology as Ozaki and Wahlsten (1992), crystals of DiI and DiA were inserted into the opposite hemispheres of the same brain. Double labelling was employed to allow differentiation of CC axons between sides in those fetuses where the CC had already crossed midplane. The primary aim of this experiment was to measure the horizontal component of CC growth between the two hemispheres to ascertain whether a gross cortical asymmetry existed, rather than the differences in growth between cortical regions.

Methods and Materials

Within the present experiment the subjects were 41 B6D2F_2 and 30 BALBc/Wah1 fetuses. The B6D2F_2 fetuses were extracted from B6D2F_1 females, the latter being obtained by a female C57BL/6J x male DBA/2J cross. The B6D2F_2 strain was used as a control group to calculate developmental age for a standardized population because they are good breeders whose offspring are genetically heterogeneous and extremely viable (Wahlsten, 1974). All mice were housed in a room with a 12 hour light-12 hour dark schedule and allowed access to food and water ad libitum. The animal cages were changed weekly and the cages containing the pregnant females were provided with nestlets for nest construction.

To obtain fetuses one male and three to five females were placed together each

day at 0800. Pregnant females were obtained by testing for vaginal plugs at 1200 and 1600 with point of conception calculated as midway between plug checks (i.e. a female with a plug detected at 1600 was considered impregnated at 1400). When a plug was found the female was removed, weighed and housed apart until the fetuses were extracted. The extraction time was calculated to achieve the desired gestational periods, which ranged from embryological day (E) 14.0 to E17.0.

To obtain the fetuses the mother was anaesthetized with sodium pentobarbital (120mg/kg, i.p. injection), the uterus extracted and the fetuses removed. The mother was then euthanized by cervical dislocation. Immediately after extraction the fetuses were placed in ice-cold 0.9% physiological saline, weighed, and then perfused intracardially. The fetuses were initially perfused with 3-5 ml of phosphate buffered saline and then with 10-15 ml 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.6). Afterwards the head was cut from the body and both were immersed in fresh fixative.

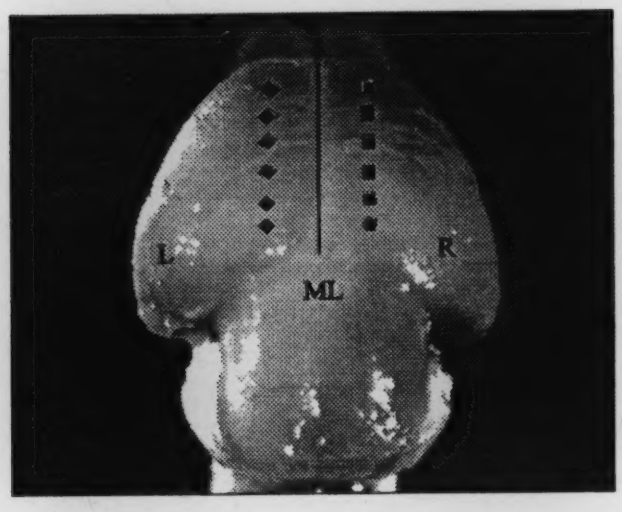
To insert the dye crystals the fetus heads were placed in a petri dish and using forceps the top of the skull was opened to expose the brain. Using a 10X graticule in the eyepiece of a dissecting microscope, four to six crystals (50-100 μm) of the lipophilic dyes (Molecular Probes Inc.) either DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) or DiA (4-(4-dihexadecylaminostyryl)-N-methyl-pyridinium iodide) were inserted into the cerebral cortex of each hemisphere with a fine pin. The number of crystals used depended on the size of the brain. For the brains of fetuses greater than 1.0 grams (g) six crystals were usually sufficient to cover the rostral caudal extent of the cerebral cortex, while fetuses weighing more than 0.6 g used five

crystals and less than 0.6 g received four. Double labelling was accomplished by inserting the crystals of DiI and DiA in opposite hemispheres (see Figure 1). Between brains, the side that received DiI or DiA was staggered so that one hemisphere did not always receive the same dye across all subjects. After crystal application the brains were removed from the skull, placed in fresh 4% paraformaldehyde and incubated in the dark at 37 C. Fetuses weighing less than 0.4 g were incubated for 3 weeks, and incubation time was increased by one week for each 0.1 g increase in fetal weight.

When the dye had fully diffused the brains were gelatinized (Livy and Wahlsten, 1996) and then sectioned using a DSK microslicer. The sections were cut serially at a thickness of 50 µm, counterstained with bis-benzimide (Sigma)(.002% in mounting medium) and stored in a solution of mounting medium (1:1 glycerol and 8% PFA at pH 10.0) at 4 C. After 24 hours the sections were mounted and coverslipped using the mounting medium solution. The slides were viewed using a 10X eyepiece in a Leitz epifluorescence microscope at a magnification of 6.3X. DiI was viewed using the standard N2 Rhodamine filters, DiA with selective FITC filters and cell staining with a B2 bis-benzimide filter.

The growth of the fibre tract was measured using the minimum or maximum horizontal distance (MD) from the main bundle (MB) to midline (ML). The minimum distance was the closest approach to midline when the axons had not crossed and the maximum distance was the greatest distance from midline after the axons had crossed. The main bundle was defined as the outer medial edge of the area where the largest collection of CC axons was found, and measurements were taken from a point just inside

Figure 1. Photograph of a dorsal view of a B6D2F$_2$ fetus brain illustrating putative dye crystal insertion sites for DiI in the right hemisphere (■) and DiA in the left hemisphere (♦).



ML: Midline

R: Right

L: Left

the leading edge (see Figure 2). The minimum distance was negative in brains where the CC had not crossed, and the maximum distance was positive in brains where the CC had proceeded into the contralateral hemisphere. Initially the maximum or minimum horizontal distance for each dye was measured in several serial sections using a graticule in the eyepiece. The largest MD (i.e. the greatest extent of growth) for each side was then noted separately and used in the analysis.

Results

Initially, the intra-observer reliability was computed using Pearson product moment correlations. Using a multiple regression equation, the first step in the analysis was to test a number of variables to determine the best predictors of a specific measurement parameter. Using this same equation the potential nonlinearity of the data was also assessed by adding a quadratic term to the equation. The variables with the most predictive power were then used in linear regression equations to produce a line of best fit. Earlier work in this laboratory had suggested that body weight was a more accurate predictor of development than embryological age (Wahlsten, 1987). A litter extracted at a specific embryological age will contain fetuses at varying stages of development. Therefore, the degree of development within a fetus may be more accurately determined using that individual's body weight as a predictor. Hence, using minimum or maximum distance as the dependent variable, body weight was used in a regression equation as an independent variable. This allowed the determination of: 1) the amount of the variance in growth (i.e. MD) that could be explained using body weight, and 2) the linearity of the relationship between the predicted measurement variable and body weight. Body weight

Figure 2. Photograph of a coronal section in a B6D2F$_2$ fetus showing the main bundle (MB), where minimum distance (MD) is the horizontal distance from the callosal main bundle (MB) to midline (ML). In this example an asymmetry existed, with CC axons from the left hemisphere having grown farther than axons from the right.

accounted for a large portion of the variance in CC growth and the relationship was linear, so it was concluded that axon growth could also predict body weight. The equation using weight as a dependent variable (estimated body weight; EBW) was calculated in each experiment and this allowed EBW to be used as a common measurement that allowed comparisons to be made between experiments that focused on different systems. In Experiment 1 the EBW was predicted separately for each strain using the average of the minimum or maximum distance for both hemispheres. The equations derived from this procedure provided estimated body weights for each side and comparisons were made between the actual and predicted body weights and between the slopes of individual regression lines using multiple paired t-tests (Ozaki & Wahlsten, 1992). The Student's t-test was used to determine if directional asymmetry was present in planned comparisons between a number of factors. The Student's t-test is commonly used to test for differences between means obtained from different conditions and can be used to test differences between correlated means, as obtained from repeated measures. Also, if needed, post-hoc contrasts could be substituted into the generic formula for the Student's t-test (Marascuilo & Serlin, 1988).

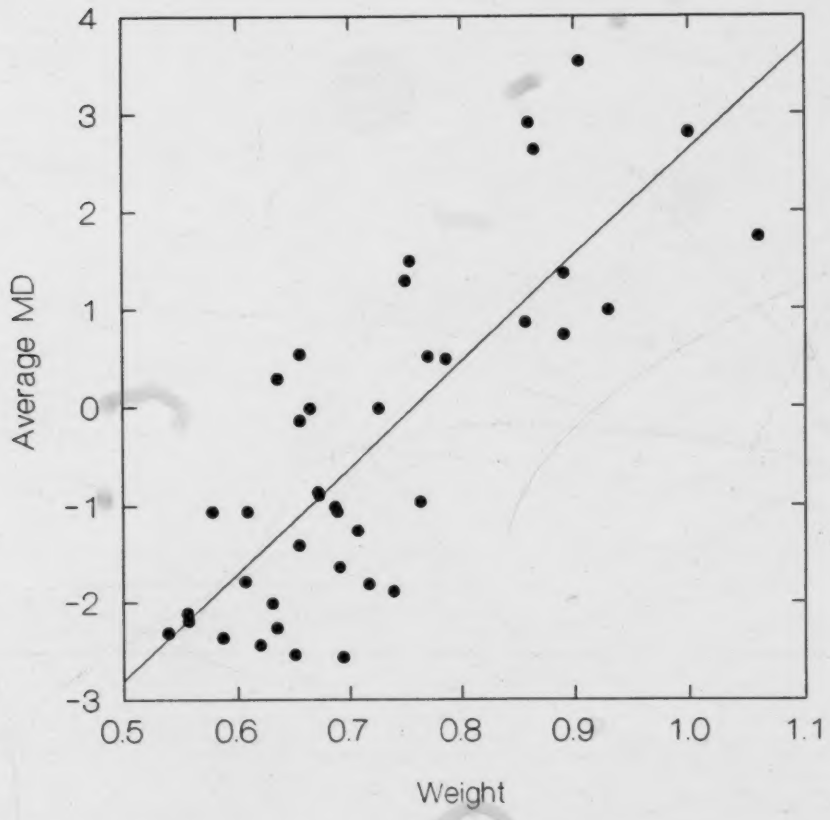
If directional asymmetry was not apparent the right-left differences were investigated for the possibility of fluctuating asymmetry (FA). A number of indices exist with which to measure FA. Palmer and Strobeck (1986) recommended that the variance of the signed differences between the sides be used, because it is more sensitive than the mean of the absolute value of the differences. Therefore, fluctuating asymmetry was

calculated as: FA=var(R-L). The variance between samples was analyzed using F-tests (Kirk, 1982) and multiple pairwise t-tests.

Intra-observer reliability was tested using 14 B6D2F$_2$ brains. Maximum distance was remeasured using the same methodology as described above. Pearson correlation coefficients were calculated separately for the MD measured with DiI (r=0.934) and DiA (r=0.875). Because the reliability for DiA appeared much lower than the correlation for DiI, the two coefficients were tested to see if they differed significantly. Each correlation was transformed into a Fisher's Z coefficient and using a z test the Z coefficients were not found to be significantly different (z=0.785, p>.01).

In B6D2F$_2$ fetuses the average minimum distance (AMD=(MDR+MDL/2)) was computed using distances from both hemispheres (collapsed across dyes) and used in a multiple regression equation with body weight (WT) as the independent variable. The resulting equation (AMD=-8.24+10.88(WT), R^2=0.639) was linear (see Figure 3) because adding a nonlinear component failed to increase goodness of fit significantly (AMD=-11.91+20.65(WT)-6.31(WT*WT), R^2=0.643). The equation for estimated body weight was then calculated by using the average minimum distance as the independent variable predicting body weight (EBW=.745+.059(AMD)). The estimated body weights for each side were obtained by inserting the right MD and the left MD into this equation as independent variables. Two paired t-tests between the EBW for each hemisphere indicated that the estimated body weights were not significantly different (t=-0.022, p>.01), and the rate of development (i.e. slopes) did not differ between hemispheres (t=-0.19, p>.01). For the BALB/cWah1 fetuses the analysis was similar to above. However,

Figure 3. A scatterplot showing the linear relationship between average minimum or maximum distance (MD) and body weight in B6D2F$_2$ fetuses.



in 10-15% of BALB/cWah1 mice the CC completely fails to cross midline; instead the CC axons form a longitudinal bundle (Probst Bundle) and grow in the ipsilateral cortex. Subjects that had a Probst Bundle were not included in the analysis because the minimum distance (MD) would not be representative of the stage of development (i.e. weight) of the fetus. For the remaining fetuses EBW for each subject was obtained by using AMD in the equations predicting EBW derived from the inbred fetuses (EBW=.742+.108(AMD)). Similar to the hybrid fetuses, the estimated body weights did not differ between hemispheres ($t=0.021$, $p>.01$), and in concordance with the hybrid fetuses the estimated rate of development (i.e. slopes) did not differ between sides ($t=-1.21$, $p>.01$).

Because directional asymmetry was not apparent, fluctuating asymmetry was investigated in both strains. The first step in analyzing FA is to test for a relationship between the magnitude of FA and character size (Palmer & Strobeck, 1986). This was tested by graphing the R-L differences against body weight. No relationship was apparent in the distributions (see Figures 4 and 5), therefore no scaling was thought to be necessary. Histograms of R-L for both strains were used to ensure the R-L differences were approximately normal and centred around zero (see Figure 6) (Markow, 1992). For the BALB/cWah1 fetuses the R-L difference appeared to exhibit a slight negative skew (see Figure 7), so a Student's t-test was performed on the R-L difference compared to the expected population difference (0). The result was not significant for either the BALB/cWah1($t=-1.474$, $p>.01$), or the $B6D2F_2$ fetuses ($t=-0.675$, $p>.01$). As per Palmer and Strobeck (1986) an ANOVA on R-L within Sides and between Strains was performed to test for the possibility of directional asymmetry (DA). No DA was present

Figure 4. A scatterplot of left maximum distance subtracted from right maximum distance (R-L) versus body weight for B6D2F$_2$ fetuses, illustrating that no relationship existed between asymmetry and character size.

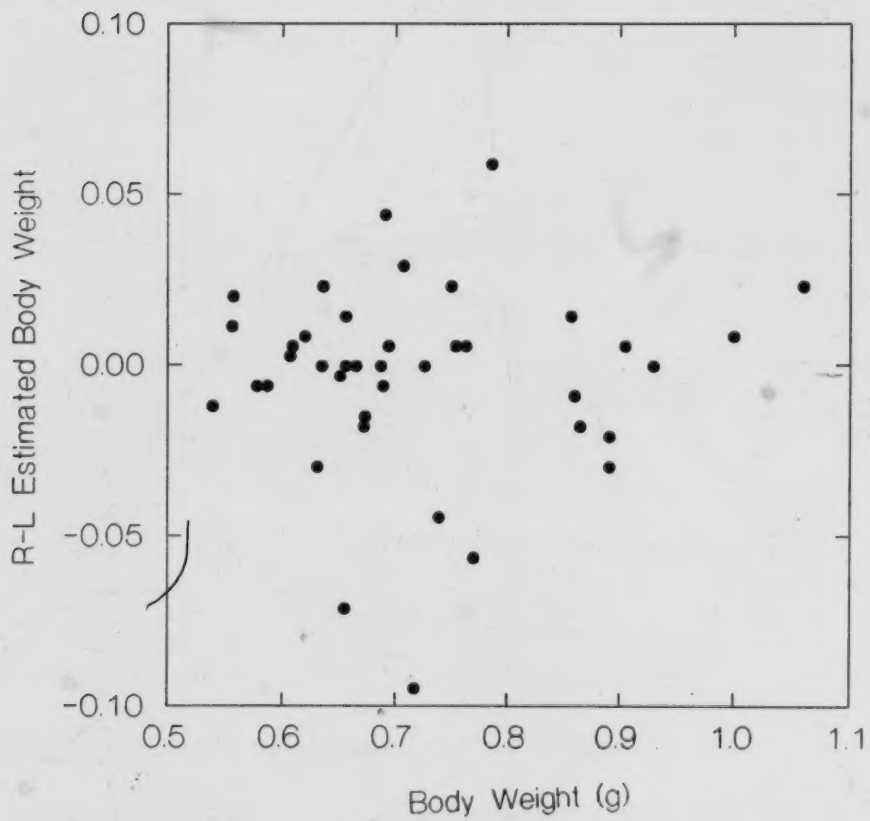


Figure 5. Scatterplot of left minimum distance subtracted from right minimum distance (R-L) against body weight for BALB/cWah1 fetuses, illustrating that no relationship existed between asymmetry and character size.

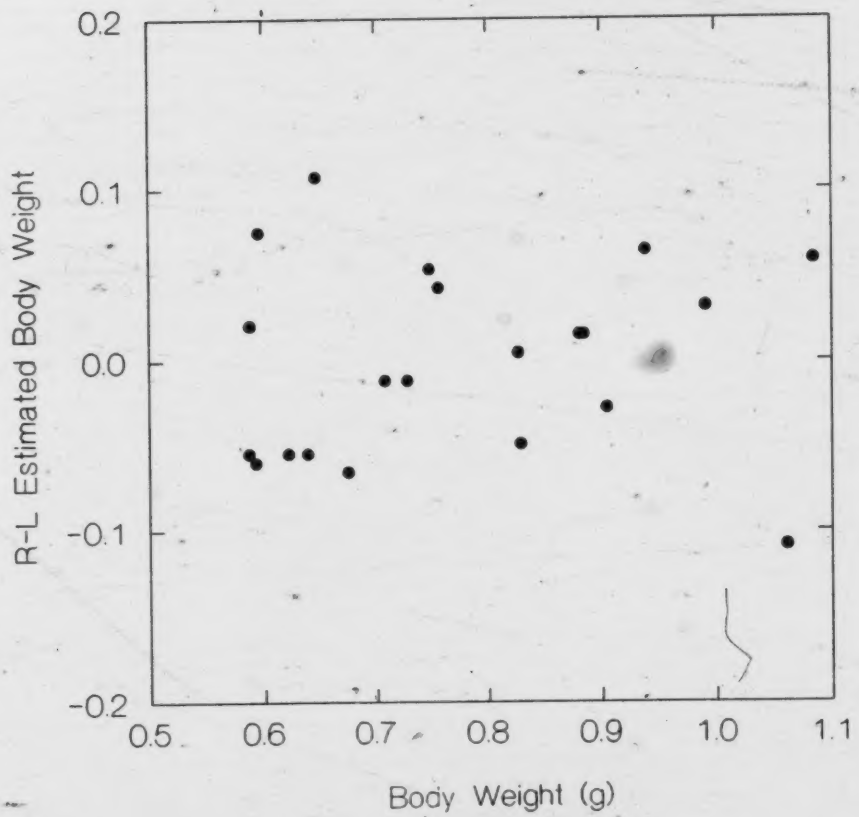


Figure 6. A histogram of the difference between right and left estimated body weight (R-L) for B6D2F$_2$ fetuses showing a normal distribution around a mean of zero, indicative of fluctuating asymmetry.

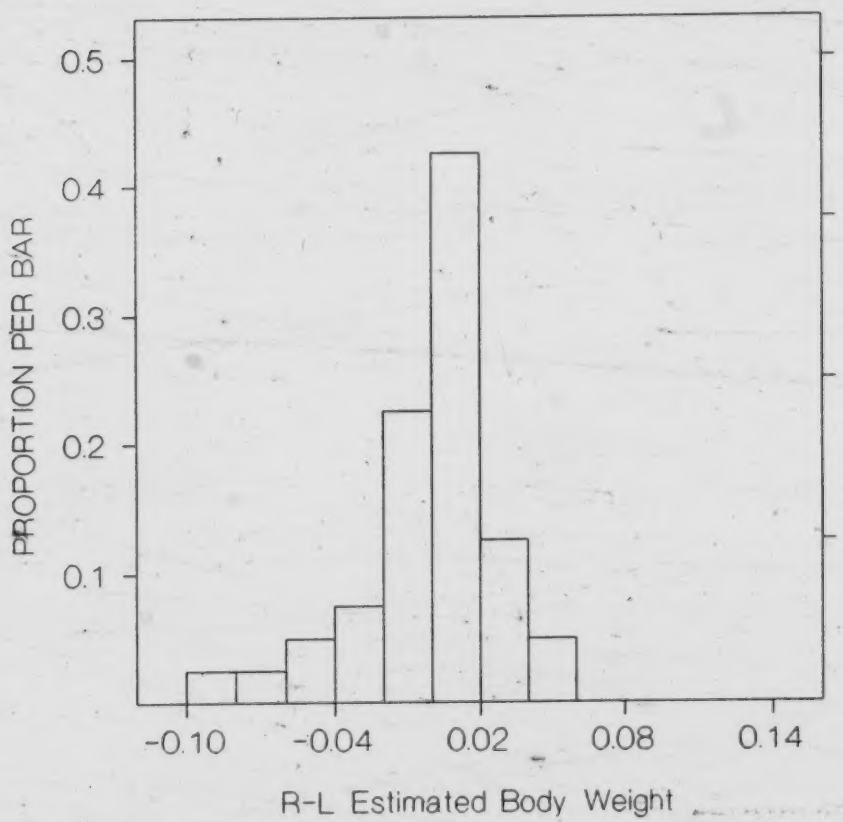
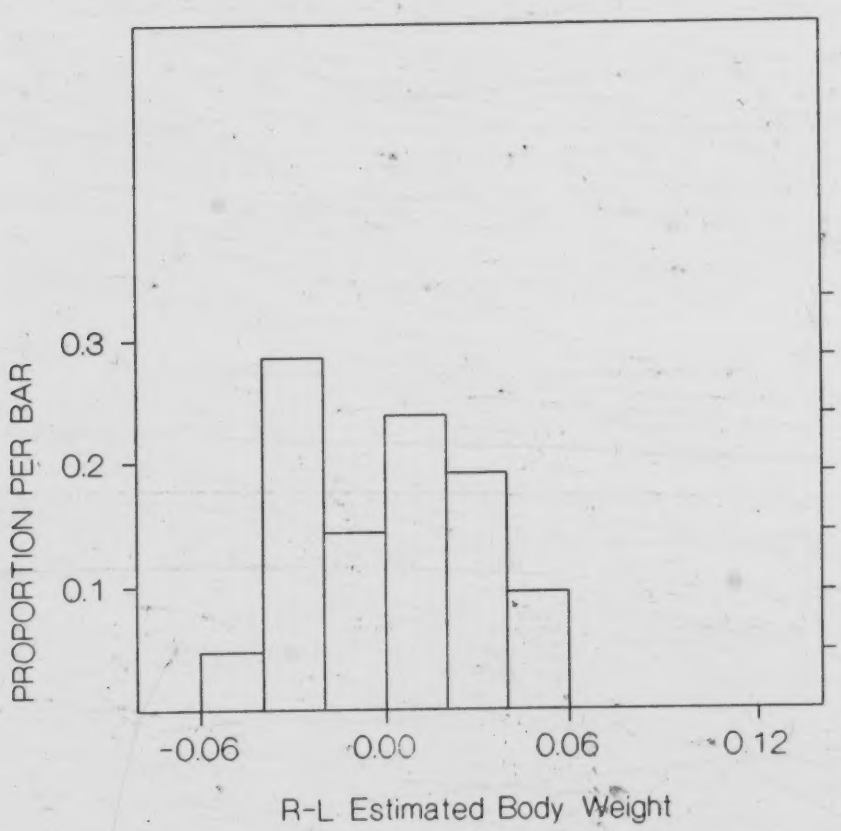


Figure 7. A histogram of the difference between right and left estimated body weight (R-L) for BALB/cWah1 fetuses showing a normal distribution around a mean of zero, indicative of fluctuating asymmetry.



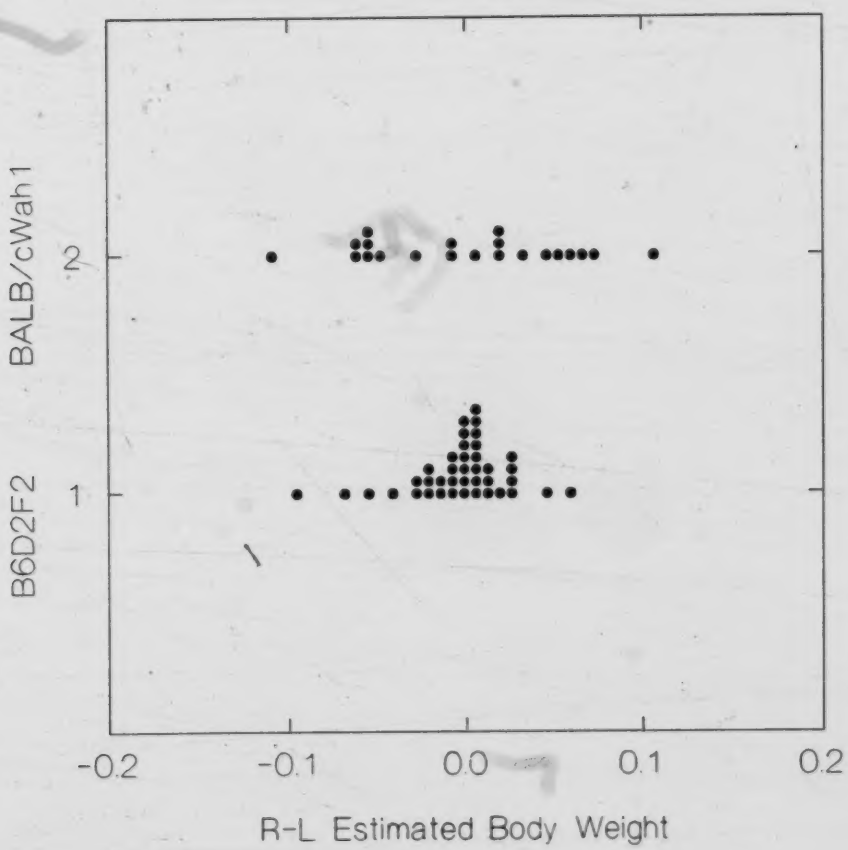
because Side was not a significant factor ($F_{(1,59)}$=0.134, p>.01), while the effect of Strain approached significance ($F_{(1,59)}$=3.99, p=.051). To ascertain if the amount of FA in each strain differed, the variance of R-L was calculated and comparisons between samples were done using an F-test (Kirk, 1982); and again the amount of FA between the B6D2F_2 (FA=0.027) and BALB/cWah1 (FA=0.054) fetuses again approached significance ($F_{(20,39)}$=2.0, p=.03).

Discussion

The present experiment yielded three primary conclusions. First, no gross directional asymmetry was apparent in the arrival of CC axons at midplane. Second, the use of multiple dye placements in a single hemisphere may have obscured subtle right-left differences in CC growth. Finally, the use of DiA presented insertion and measurement difficulties that were not apparent with the use of DiI.

The results from this experiment indicated that no gross directional asymmetry was evident in the development of the CC. However, individual asymmetry was apparent in most subjects. These individual differences appeared to be representative of fluctuating asymmetry (FA). The amount of FA was greater in the BALB/cWah1 fetuses. But as can be seen in Figure 8, even though the variance of the R-L differences is greater for the inbred fetuses, the spread of R-L is approximately equal between the two strains. This may explain why the strain differences failed to reach significance at the .01 level. Despite the greater FA of the BALB/cWah1 fetuses the lack of a significant difference between the amount of cortical growth between strains is consistent with Ozaki and Wahlsten (1993). In a study of fetal axon growth in B6D2F_2, BALB/cWah1 and 129/ReJ

Figure 8. A density plot of R-L differences in estimated body weight for both the B6D2F$_2$ and BALB/cWah1 fetuses. This figure shows the larger R-L differences evident in the BALB/cWah1 fetuses, even though the range of R-L differences is approximately equal between the strains.



mice the authors did not find that the growth and arrival of CC axons differed significantly between strains.

In an earlier work Ozaki and Wahlsten (1992) found a rostro-caudal gradient in the rate of cortical development. The fact that different areas of the brain develop at different rates presents the possibility that an asymmetrical rate of development could exist between hemispheres that differs from cortical region to cortical region. That is, within a brain the frontal cortex of the left hemisphere could develop more rapidly than the frontal cortex of the right; while, the temporal cortex of the right hemisphere could have a similar advantage over the temporal cortex of the left. A difficulty that arose from the methodology employed by this study was the loss of this intra-hemispheric information. Because the dye crystals were applied in a rostro-caudal strip it was not possible to differentiate between the axons growing from the different cortical areas. The dye placements needed to be more discrete if asymmetry between different cortical areas was to be investigated.

Another difference in methodology that arose between Experiment 1 and Ozaki and Wahlsten (1992) was how the main bundle of CC axons was defined, and consequently how the distances from the main bundle to midplane were measured. Experiment 1 used a larger collection of axons to define the main bundle, and as can be seen in Figure 2, axon growth is apparent beyond the labelled main bundle. This growth would have been included in the Ozaki and Wahlsten definition of main bundle. Therefore, in fetuses of similar body weight the distance between the ipsilateral callosal bundle and midplane in Experiment 1 may be overestimated when compared to the same

distance measured by the method of Ozaki and Wahlsten.

In the present experiment double labelling within a brain was designed to allow measurement of CC symmetry at many stages of development. However, during the course of the study several limitations of using DiA became apparent. Initially, crystal placement in the cortical mantle should be as equal as possible between sides, both in the quantity and location of the dye. Both these goals were difficult to achieve because while DiI is a small compact crystal, the crystalline form of DiA is more similar to a flake. The result of this disparity in form was that DiI could be applied in distinct regions and during cutting the size of the crystal could be quantified by counting the number of brain slices where it was visible. The DiA was not amenable to precise placement as it crumbled when placed on the surface of the cortex. This resulted in dye placements that were more diffuse and extremely difficult to quantify. During the measurement process another dilemma that became apparent was the difficulty in distinguishing between DiI and DiA when the two became intermingled. To help differentiate between the dyes a bandpass, DiA-specific, filter was used. Nevertheless, the amount of fluorescence visible using the bandpass filter was insufficient to visualize the distal portions of the main bundle, therefore the full extent of CC growth was difficult to estimate. Also, the more diffuse DiA insertion may have led to less intense labelling and combined with the difficulty in viewing contralateral CC axons, may have had a significant impact on the validity of the results. Specifically, the amount of FA could have been an artifact of unequal axonal fluorescence rather than uneven axonal growth, especially in measurements of the contralateral hemisphere.

EXPERIMENT 2 - DISCRETE DYE INJECTIONS

Introduction

This purpose of this study was twofold: To refine the techniques and hence increase the accuracy of Experiment 1 and to test whether the degree of asymmetry may differ between cortical regions. Ozaki and Wahlsten (1992) had shown that the rates of growth between cortical areas within a hemisphere were not equal, i.e. a rostral-caudal gradient existed. Because there is a differing rate of growth between cortical areas, dye injections were planned for the frontal, temporoparietal and occipital cortices. The temporal and parietal cortices were not injected separately because Ozaki and Wahlsten (1992) had shown that the rate of growth between these two regions did not differ greatly. While Experiment 1 had shown that within a brain the total amount of CC axon growth was generally unequal between hemispheres, the pattern of dye placement sites made it extremely difficult to test for right-left differences between cortical areas. To increase the accuracy of the dye injection procedure Experiment 2 utilized a mechanical delivery system. The fetus heads were placed on a stereotaxic stage, which ensured that placement sites were organized symmetrically along either side of ML and that different cortical areas could be precisely located. Furthermore, instead of dye crystals, DiI was applied as a solution from a picospritzer. This helped to ensure that the amount of dye injected at each site was equal.

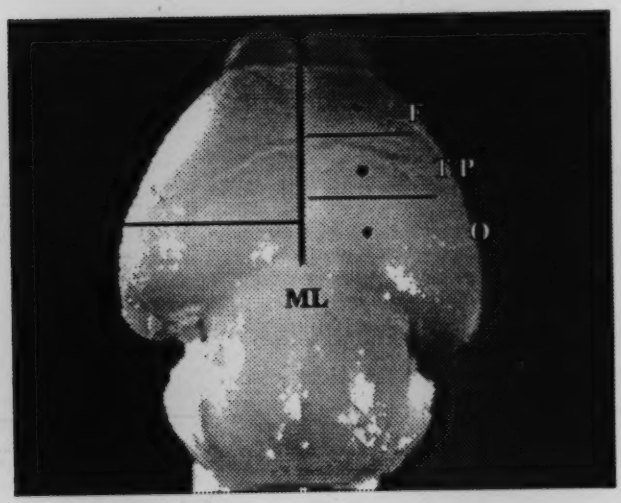
Methods and Materials

The subjects were 12 B6D2F$_2$ and 8 BALB/cWah1 mouse fetuses. The subjects weighed less than 0.62 g and were selected specifically to ensure that CC axons had not

yet crossed midplane. The fetuses were obtained and perfused intracardially using the methods described above.

Initially the heads were placed in a petri dish under a dissecting microscope and the dorsal surface of the of the brain was exposed by opening the skull using forceps. The brain was left in the skull and the entire head was then glued to a plexiglass stage mounted on a David Kopf stereotaxic instrument. The brain was viewed with a dissecting microscope to ensure the skull had been placed such that midline was parallel to a line that bisected the stage. The carbocyanine dye solution FAST DiI (1,1'dioleyl-3,3,3',3'-tetramethylindocarbocyanine methanesulfonate, in oil) (Molecular Probes, Inc.) was drawn into a glass micropipette with a tip of 0.05 mm. The pipette was fastened to the stereotaxic and then further connected to a picospritzer (General Valve Corp.) infused with industrial grade nitrogen and maintained at 30psi. The pipette tip was lowered to the rostral end of midline just caudal to the olfactory bulbs and a reading on the vernier scale was taken. The pipette was then moved to the lateral edge of the hemisphere and another reading taken. Finally, the pipette tip was moved to the caudal end of midline and the distance on the vernier scale was noted. The mediolateral placement points were obtained by dividing the distance from midline (ML) to the lateral edge of the hemisphere in half, then adding and subtracting this number from the original ML measurement. The anterior-posterior (AP) dimensions were calculated by dividing the rostral-caudal distance into thirds. This corresponded to the frontal, temporoparietal and occipital cortices, and injections were planned for the midpoint of each cortex (see Figure 9). The dye was injected by lowering the pipette tip until it just pierced the cortical mantle and

Figure 9. Dorsal view of a B6D2F$_2$ fetus brain showing the measurement parameters for midline length, hemisphere width, cortical areas and putative injection sites for each cortical area (•).



F: frontal cortex

TP: temporoparietal cortex

O: occipital cortex

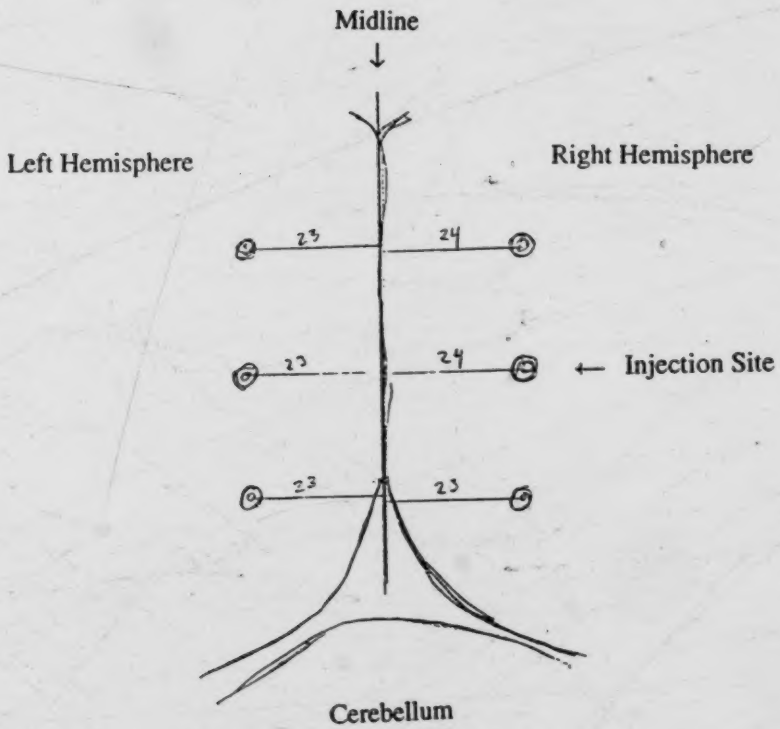
ML: midline

then injecting a single pulse of .01µl for 4msec at 10psi. The pipette was immediately raised and moved to the next injection site. Once all three regions in both hemispheres had been injected the head was removed from the stage and replaced in fixative. The brains were left in the skulls and incubated at 37 C for 4 weeks.

The symmetry of the mediolateral distance between dye injection sites was ensured by tracing a dorsal view of the brain using a 10X eyepiece in a Dialux microscope at 2.5X magnification and a Leitz Weitzlar tracing device. The heads were placed in a concave microscope slide illuminated from below and dorsal view tracings were made of midline (from the olfactory bulbs to the rostral edge of the cerebellum), the injection site and surrounding area of diffusion. Using a ruler a straight line was drawn along midline and perpendicular to this a line was drawn to the centre of the dye injection site. The distance from injection site to ML was measured (mm) using a ruler (see Figure 10). Brains that were not injected symmetrically were not used in the final analysis because accurate dye placements were crucial to successfully measuring the symmetry of axon growth.

After four weeks a control brain that had been injected the same day was cut to verify that the dye had diffused to the full extent of CC projections. Complete dye transport was confirmed when growth cones were visible because this signalled the furthest extent of axon growth. After confirmation the experimental brains were removed from the skulls, gelatinized, sectioned and mounted using the same methodology as described above. The slides were viewed at 10X magnification using a Leitz epifluorescence microscope with rhodamine filters and cell staining with a B2 bis-

Figure 10. Tracing of a dorsal view of a B6D2F$_2$ brain showing the symmetry of the dye injection sites.



benzimide filter.

For all dye injection sites in each hemisphere the section that contained the centre of the injection site was used to begin calculating axon length. A composite diagram was constructed by tracing the extent of CC development in successive sections, from the intermediate zone (radial glia) until the medial extent of the main bundle was reached. Each sheet of paper had a circle drawn on it that represented the view through the ocular and had a known size. The extent of the CC for each section was traced on a sheet of paper using a fixed location (the dye injection site) as a starting point. The composite diagrams were constructed on the same sheet of paper, but to avoid experimenter bias the right and left sides of the same subject were not diagrammed at the same time. Afterwards, a dot was placed in the centre of each traced DiI bundle and the points were connected by a line running from the injection site to the leading edge of the main bundle. Using Sigma Scan (Jandel Scientific) the distance from the insertion site to the main bundle (ISMB) was calculated by measuring this line (mm) (see Figure 11). The anterior-posterior distance was calculated by multiplying the number of sections used, minus one, by the thickness of each section. This value was then added to the mediolateral measurement. This measurement was similar to the ISMB distance measured in Ozaki and Wahlsten (1992).

Results

Reliability was tested by remeasuring all 12 B6D2F$_2$ brains. A Pearson correlation matrix was constructed and the r values ranged from 0.812 to 0.904 (see Table 1).

Figure 11. A composite diagram of serial coronal sections, illustrating the mediolateral extent of DiI fluorescence (a to b) measured by the insertion site to main bundle (ISMB) method of Experiment 2. The ISMB distance was calculated by measuring the line traced through the outlined dye bundles.

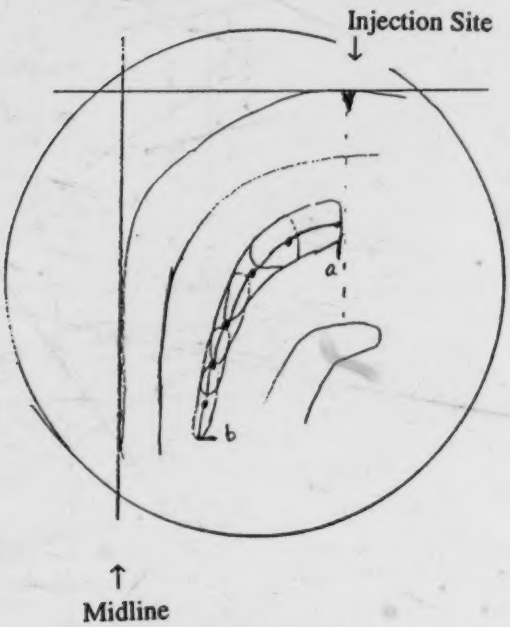


Table 1

Correlations of reliability measurements for ISMB in Experiment 2.

	ISMB Measurement					
ISMB Reliability	LF[1]	RF[1]	LTP[2]	RTP[2]	LO[3]	RO[3]
LFREL[4]	.876					
RFREL[4]		.899				
LTPREL[5]			.904			
RTPREL[5]				.903		
LOREL[6]					.870	
ROREL[6]						.812

[1] lf/rf: left frontal/right frontal ismb

[2] ltp/rtp:left temporoparietal/right temporoparietal ismb

[3] lo/ro: left occipital/right occipital ismb

[4] lfrel/rfrel: left frontal ismb reliability/right frontal ismb reliability

[5] ltprel/rtprel: left temporoparietal reliability/right temporoparietal reliability

[6] lorel/rorel: left occipital ismb reliability/right occipital ismb reliability

Multiple regression was performed on the data for each cortical area to establish the linearity of the data and determine if the slope of the regression equations was similar to those obtained by Ozaki and Wahlsten (1992). The data were considered linear for each cortical area and not transformed because the addition of a quadratic term to the multiple regression equation failed to improve goodness of fit significantly (see Table 2).

Table 2

Regression equations for each cortical area in Experiment 2, showing the non-significant increase in goodness of fit (R^2) with the addition of the quadratic term.

Cortex	Equation	R^2	F	p
Frontal	avgf[1]=-5.19+11.06(wt[4])	0.884		
	avgf[1]=-2.76+2.82(wt)+6.8(wt*wt)	0.884	0.0	>.01
Temporoparietal				
	avgtp[2]=-3.16+7.0(wt)	0.880		
	avgtp[2]=9.69+-36.6(wt)+36.9(wt*wt)	0.884	0.53	>.01
Occipital				
	avgo[3]=-1.98+4.7(wt)	0.881		
	avgo[3]=-21.34+70.32(wt)-55.56(wt*wt)	0.905	2.08	>.01

[1] avgf: average frontal ismb distance in millimetres

[2] avgtp: average temporoparietal ismb distance in millimetres

[3] avgo: average occipital ismb distance in millimetres

[4] wt: weight

Using multiple t-tests the regression equations obtained for each cortical area of the B6D2F$_2$ fetuses, using body weight to predict axon distance (ISMB), were tested against the slopes of the regression equations obtained by Ozaki and Wahlsten (Marascuilo & Serlin, 1988). None of the slopes differed significantly (see Table 3) and plots comparing the slope of insertion site to main bundle (ISMB) distance versus weight

Table 3

Comparison of the regression equations obtained by Ozaki and Wahlsten (1992) and Experiment 2.

Cortex	Equation	r^2	t	p
Frontal				
Ozaki & Wahlsten	ismb[1]=-4.42+10.75(wt[5])	0.92		
Experiment 2	avgf[2]=-5.19+11.06(wt)	0.884	1.69	>.01
Temporoparietal				
Ozaki & Wahlsten	ismb=-3.06+7.58(wt)	0.89		
Experiment 2	avgtp[3]=-3.16+7.0(wt)	0.884	1.4	>.01
Occipital				
Ozaki & Wahlsten	ismb=-2.38+6.21(wt)	0.92		
Experiment 2	avgo[4]=-1.98+4.7(wt)	0.881	2.9	>.01

[1] ismb: measurement of insertion site to main bundle for Ozaki & Wahlsten

[2] avgf: average frontal ismb distance in millimetres

[3] avgtp: average temporoparietal ismb distance in millimetres

[4] avgo: average occipital ismb distance in millimetres

[5] wt: weight

for both Experiment 2 and Ozaki and Wahlsten confirmed the similarity between the slopes (see Figures 12, 13, 14). Therefore, ISMB from the present study was inserted into the equations of Ozaki and Wahlsten to determine the right and left estimated body

Figure 12. A plot of frontal cortex axon distance (ISMB) travelled (mm) versus weight, showing the similarity of the slopes for the line of best fit measured in Experiment 2 and predicted using the regression equation of Ozaki and Wahlsten (1992).

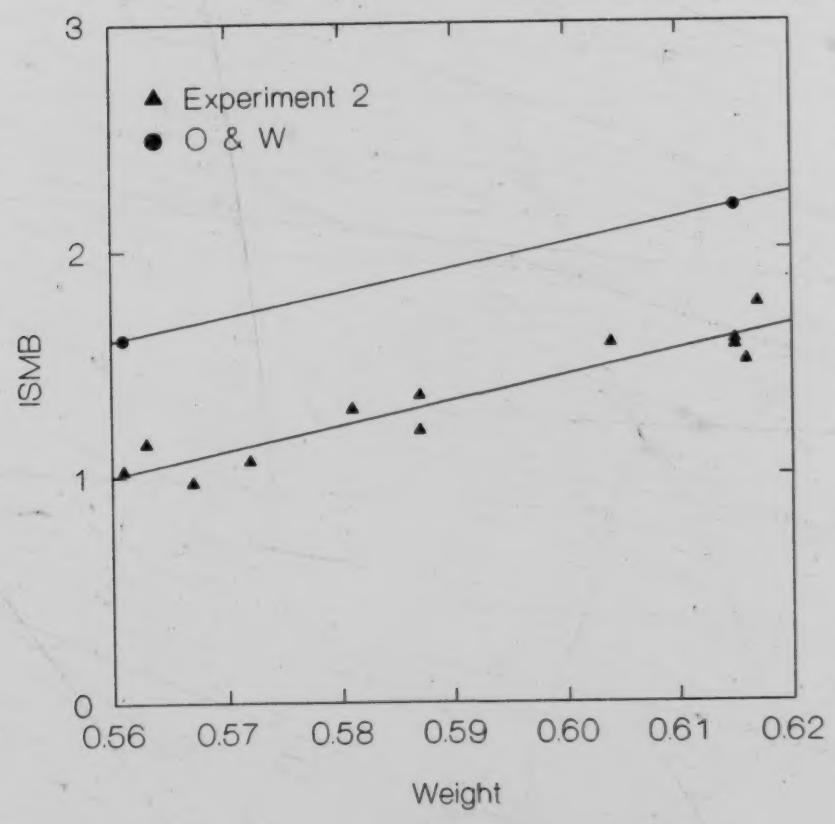


Figure 13. A plot of temporoparietal cortex axon distance (ISMB) travelled (mm) versus weight, showing the similarity of the slopes for the line of best fit measured in Experiment 2 and predicted using the regression equation of Ozaki and Wahlsten (1992).

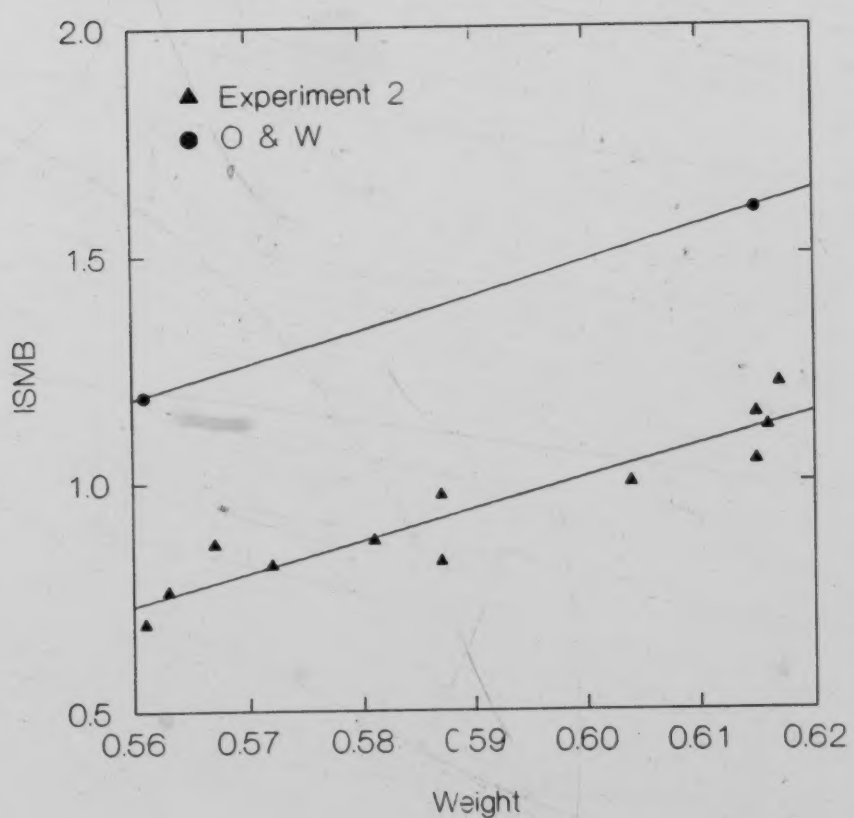
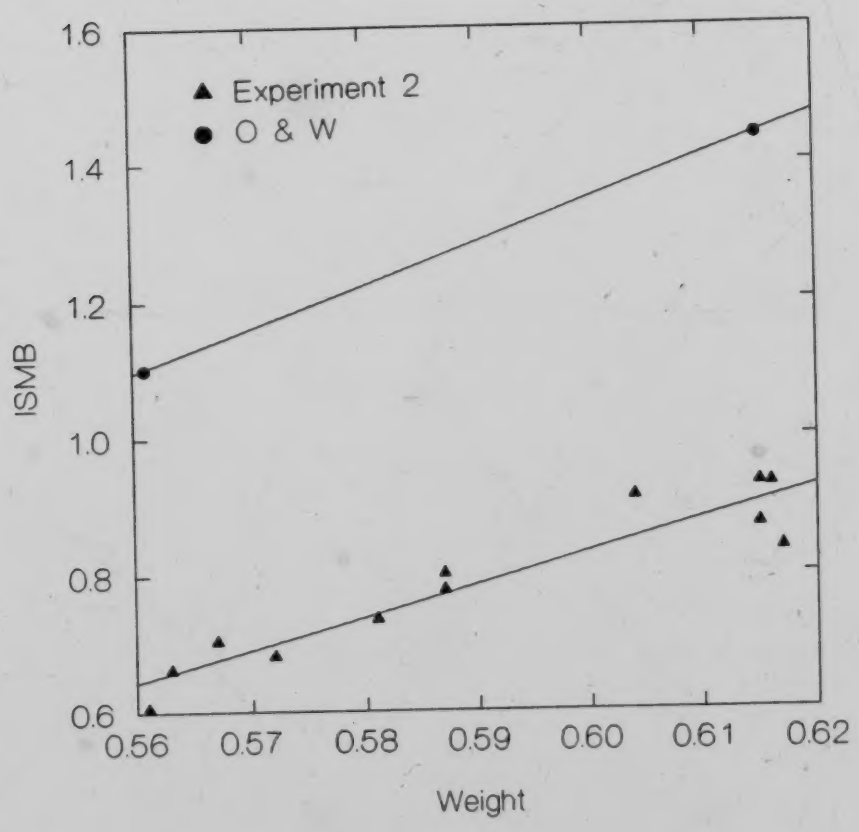


Figure 14. A plot of occipital cortex axon distance (ISMB) travelled (mm) versus weight, showing the similarity of the slopes for the line of best fit measured in Experiment 2 and predicted using the regression equation of Ozaki and Wahlsten (1992).



weights for each subject.

Theoretically, if both sides of an organism develop at the same rate then the estimated body weight (EBW) for each side should be equal. To this end multiple pairwise t-tests were used to determine if the EBW differed between sides. The right and left EBW was not significantly different between the left and right sides for any cortical area in either strain (see Table 4). However, the variance of R-L differences was uniformly greater in the inbred fetuses than the hybrid fetuses. Because directional asymmetry was not evident, but a difference in the magnitude of right-left differences was apparent, the possibility of fluctuating asymmetry was investigated.

Similar to above, the magnitude of the differences between sides for each cortex was charted against body weight to determine if the difference varied with size. For the B6D2F_2 fetuses R-L appeared to increase as weight increased in the frontal cortex (see Figure 15). To investigate further this relationship (R+L)/2 was plotted against body weight (Palmer and Strobeck, 1986). This distribution appeared more random, showing a similarity to the distribution of R-L for the temporoparietal and occipital cortices (see Figures 16, 17). Therefore, scaling was not done. Histograms of R-L differences for each cortex showed that the distributions were centred around zero and pairwise t-tests indicated that none of the distributions were centred significantly far from 0 (see Table 5).

Figure 15. A scatterplot of right estimated body weight minus left estimated body weight (R-L) versus weight for the frontal cortex of B6D2F$_2$ fetuses, illustrating the possible increase in asymmetry as a function of character growth (i.e. body weight).

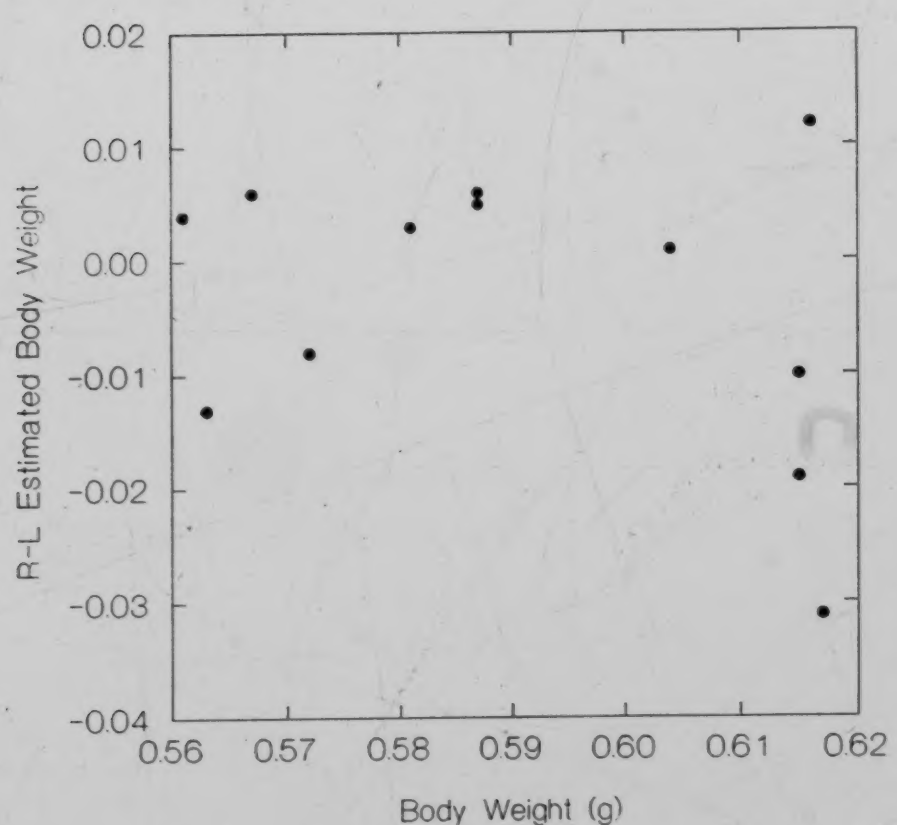


Figure 16. A scatterplot of right estimated body weight minus left estimated body weight (R-L) versus weight for the temporoparietal cortex of B6D2F$_2$ fetuses. This figure shows that no relationship existed between character size (i.e. body weight) and asymmetry.

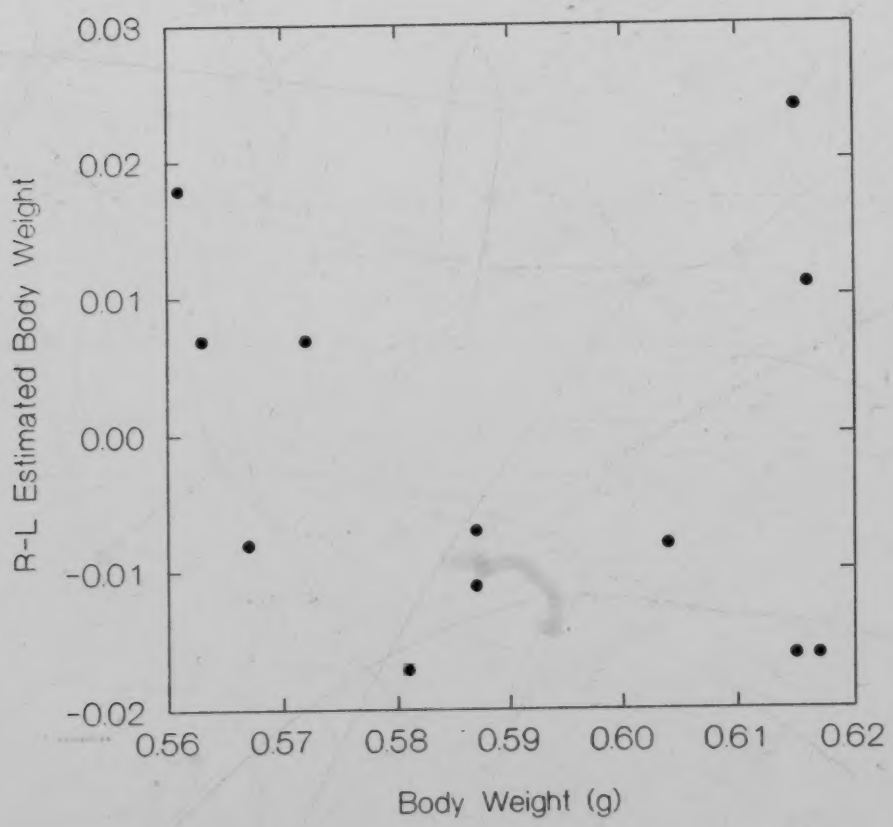


Figure 17. A scatterplot of right estimated body weight minus left estimated body weight (R-L) versus weight for the occipital cortex of B6D2F$_2$ fetuses, illustrating that no relationship existed between asymmetry and character size.

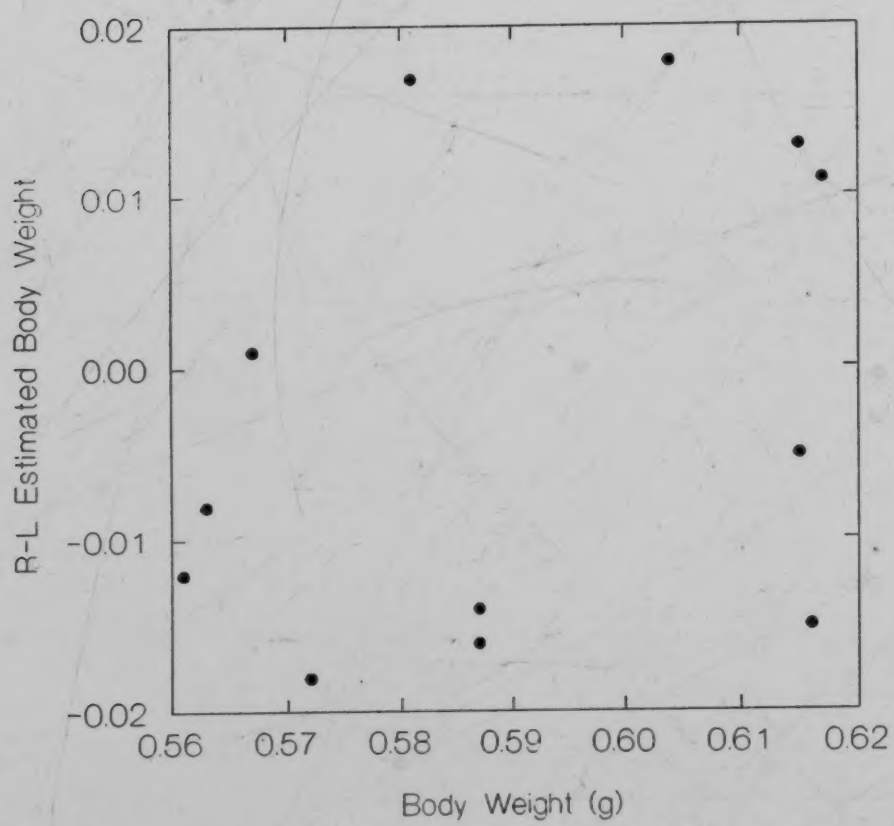


Table 4

Comparison of the right and left estimated body weights, for each cortical area, in both B6D2F$_2$ and BALB/cWah1 fetuses.

Strain	Cortex	Mean Difference	t	p
B6D2F$_2$				
	frontal	-0.004	-.0983	>.01
	temporoparietal	-0.001	-0.308	>.01
	occipital	-0.002	-0.545	>.01
BALB/cWah1				
	frontal	0.001	0.13	>.01
	temporoparietal	0.001	0.19	>.01
	occipital	-0.003	-0.463	>.01

Table 5

Differences between the sample mean, obtained by subtracting left estimated body weight from right estimated body weight for each cortical area, and the expected population mean (0), for B6D2F$_2$ fetuses.

Cortex	Mean Difference	t	p
Frontal	-0.04	-1.004	>.01
Temporoparietal	0.001	0.327	>.01
Occipital	0.002	0.589	>.01

However, partly due to the small sample size, the histograms of R-L differences did not appear to fit closely the curve of a normal distribution (see Figures 18, 19, 20). To confirm the absence of any directional asymmetry that would confound the analysis of FA, an ANOVA was conducted using Strain as the between factor and Side and Cortex as within factors (Swaddle, Witter & Cuthill, 1994). Similar to Experiment 1, directional asymmetry was not evident because Side was not significant ($F_{(1,18)}$=0.574, p>.01). Strain of animal approached significance ($F_{(1,18)}$=2.815, p=.11), while significant results were obtained for the Cortex term ($F_{(2,36)}$=84.315, p<.01) and the Strain by Cortex interaction ($F_{(2,36)}$=15.42, p<.01)(see Table 6). When R-L was plotted against weight for the BALB/cWah1 fetuses a similar tentative relationship between the magnitude of the side difference and body weight was seen, but for the only temporoparietal region (see Figure 21). Graphs of the frontal and occipital cortices failed to demonstrate this pattern (see Figures 22 and 23) and similar to above when (R+L)/2 was plotted against weight for the

Figure 18. A histogram of the difference between right and left estimated body weight (R-L) for the frontal cortex of B6D2F$_2$ fetuses. The histogram shows a normal distribution, which is indicative of fluctuating asymmetry.

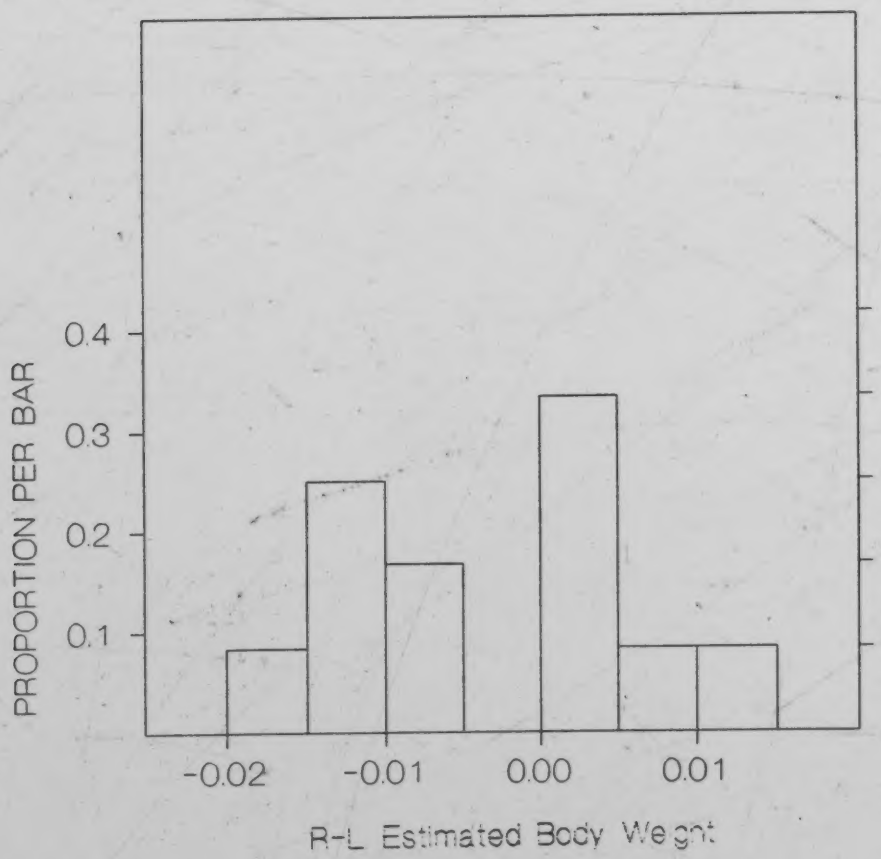


Figure 19. A histogram of the difference between right and left estimated body weight (R-L) for the temporoparietal cortex of B6D2F$_2$ fetuses. The distribution is centred around zero but due to the small sample size, lacks the characteristic normal curve.

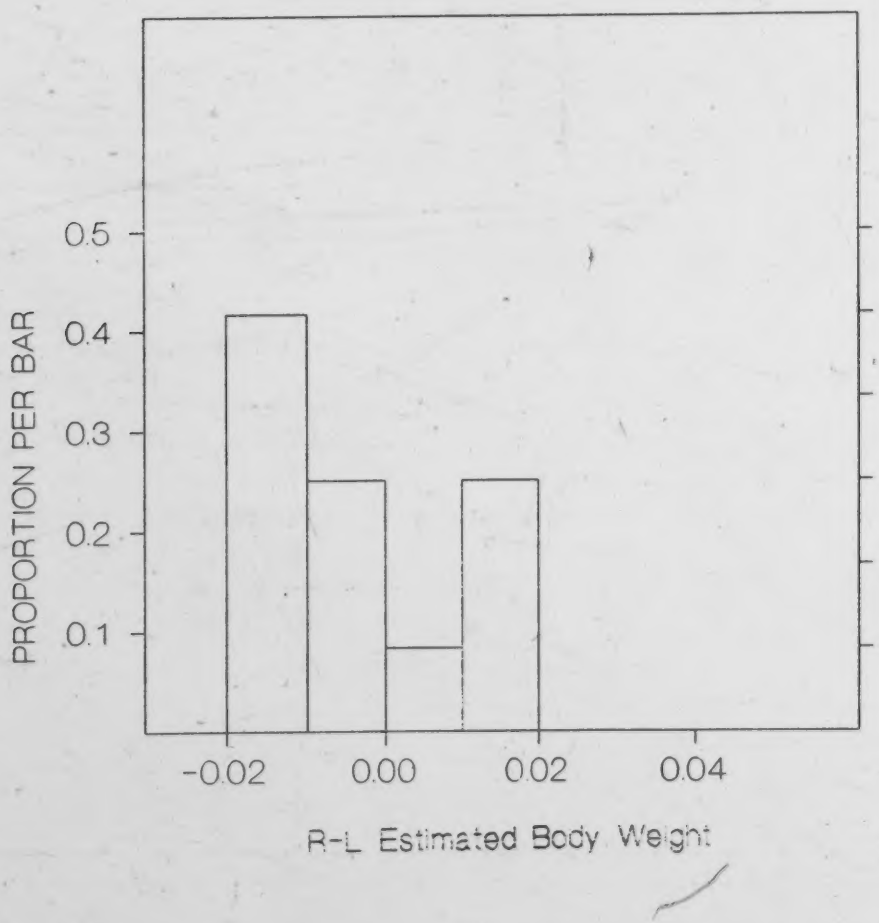


Figure 20. A histogram of the difference between right and left estimated body weight (R-L) for the occipital cortex of B6D2F$_2$ fetuses. The figure illustrates how the small sample size has produced a positively skewed distribution.

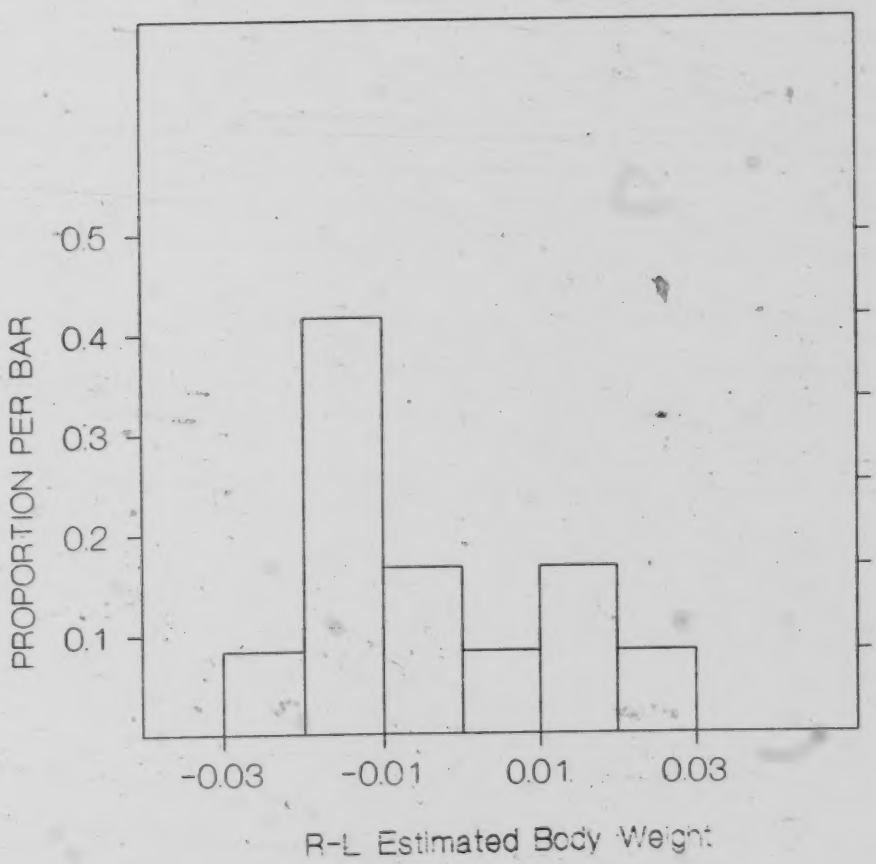


Figure 21. A scatterplot of right estimated body weight minus left estimated body weight (R-L) versus weight for the temporoparietal cortex of BALB/cWah1 fetuses. This figure illustrates the tentative relationship detected between asymmetry and character size.

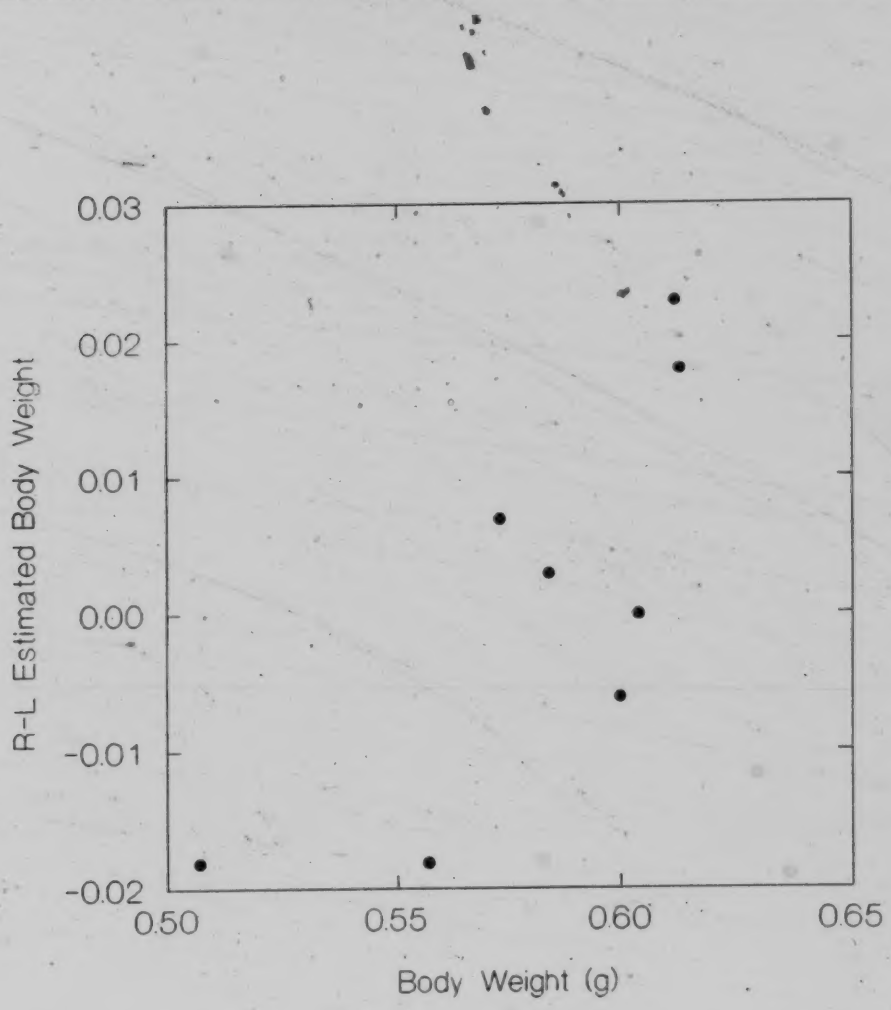


Figure 22. A scatterplot of right estimated body weight minus left estimated body weight (R-L) versus weight for the frontal cortex of BALB/cWah1 fetuses. This figure illustrates the lack of relationship between character size and asymmetry.

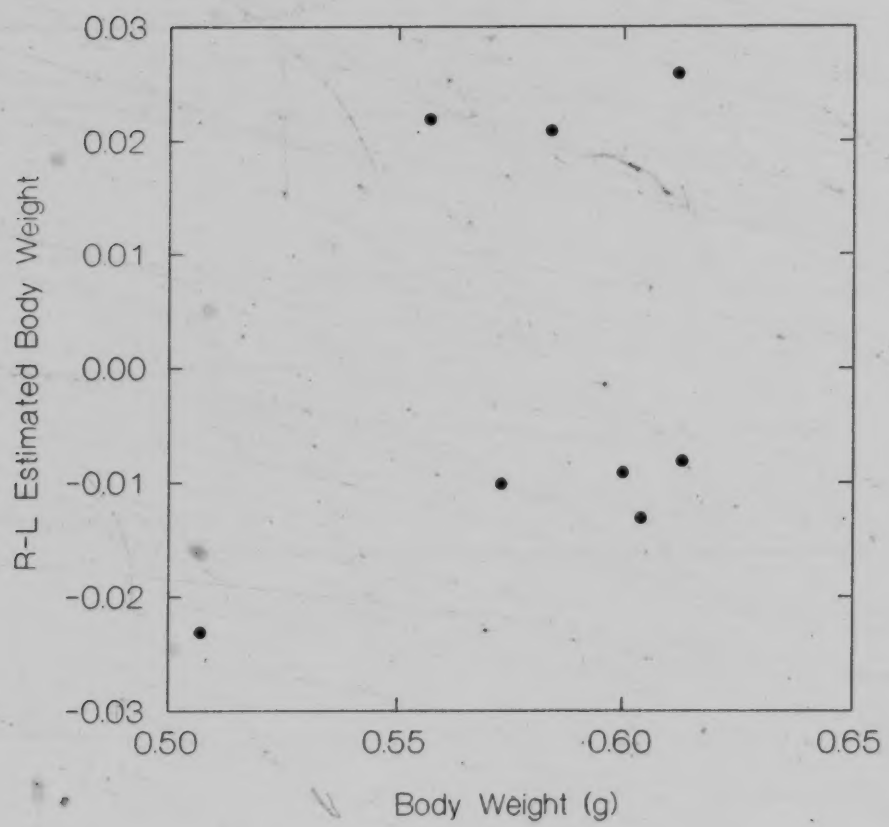


Figure 23. A scatterplot of right estimated body weight minus left estimated body weight (R-L) versus weight for the occipital cortex of BALB/cWah1 fetuses. This figure illustrates the lack of relationship between character size and asymmetry.

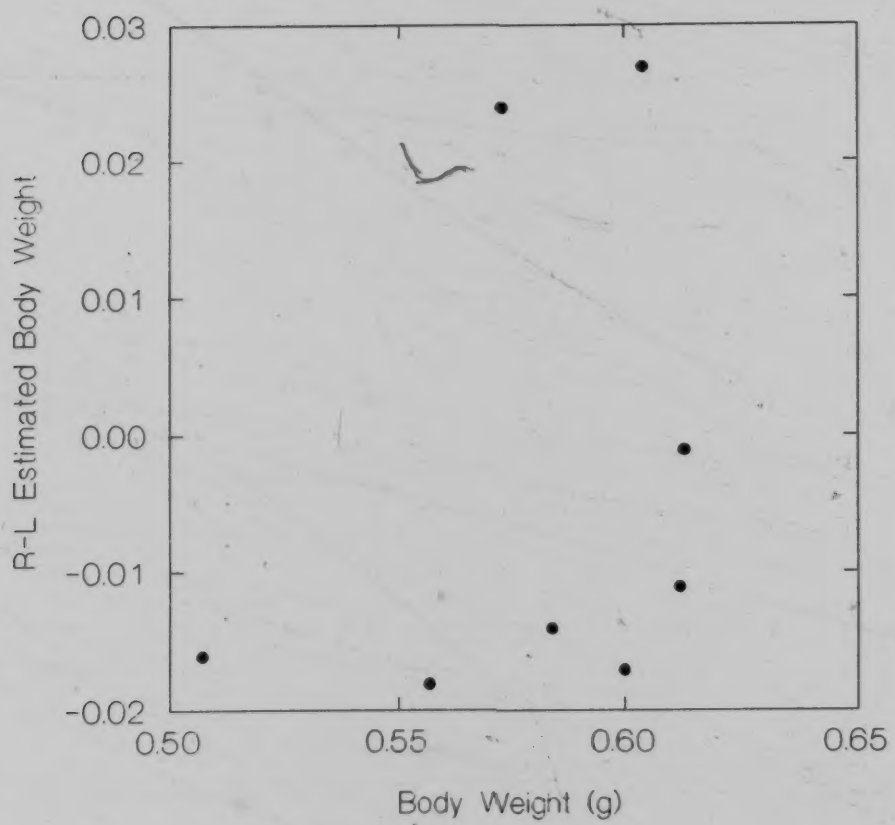


Table 6

Table of results for between Strains, within Side and Cortex ANOVA of ISMB for Experiment 2.

Source	Sum of Squares	df	Mean Square	F	p
Strain	0.422	1	0.422	2.815	.11
Error	2.698	18	0.15		
Side	0.004	1	0.004	0.574	>.01
Side x Strain	0.003	1	0.003	0.413	>.01
Error	0.121	18	0.007		
Cortex	3.153	2	1.576	84.315	<.01
Cortex x Strain	0.577	2	0.288	15.42	<.01
Error	0.673	36	0.019		
Side x Cortex	0.002	2	0.001	0.096	>.01
Side x Cortex x Strain	0.003	2	0.002	0.183	>.01
Error	0.322	36	0.009		

temporoparietal cortex no relationship between asymmetry and character size was seen. Therefore, it appeared scaling was not necessary. Similar to above, histograms of the difference between right and left confirmed the characteristic distribution of FA for each cortical area. Nevertheless, for each area the mean of the distribution was tested against the expected population mean (0) to confirm the appearance of FA. As above, none of the cortical areas differed significantly from 0 (see Table 7).

Table 7

Differences between the sample mean, obtained by left estimated body weight from right estimated body weight for each cortical area, and the expected population mean (0) in BALB/cWah1 fetuses.

Cortex	Mean Difference	t	p
Frontal	-0.001	-0.111	>.01
Temporoparietal	-0.001	-0.211	>.01
Occipital	0.003	0.496	>.01

Fluctuating asymmetry for each cortical region in both strains was calculated as the variance of R-L. To determine if the amount of FA differed between samples an F-test was used (Kirk, 1982). The comparison of FA between the strains indicated that the amount of FA was greater in the BALB/cWah1 fetuses, but the difference failed to reach significance ($F_{(7,11)}$=2.67, p=.14). Analysis of FA between cortices within each strain also failed to produce any significant differences (see Table 8).

Discussion

The results from the present experiment support the conclusions of Experiment 1 that directional asymmetry is not evident in the prenatal growth of CC axons. The estimated body weights obtained for each side failed to differ significantly in any cortical area. This indicated that, on average, the CC axons for each cortex had grown approximately the same distance in each hemisphere. However, individual asymmetry was still very common in the developing mouse fetus, with each cortical region showing

Table 8

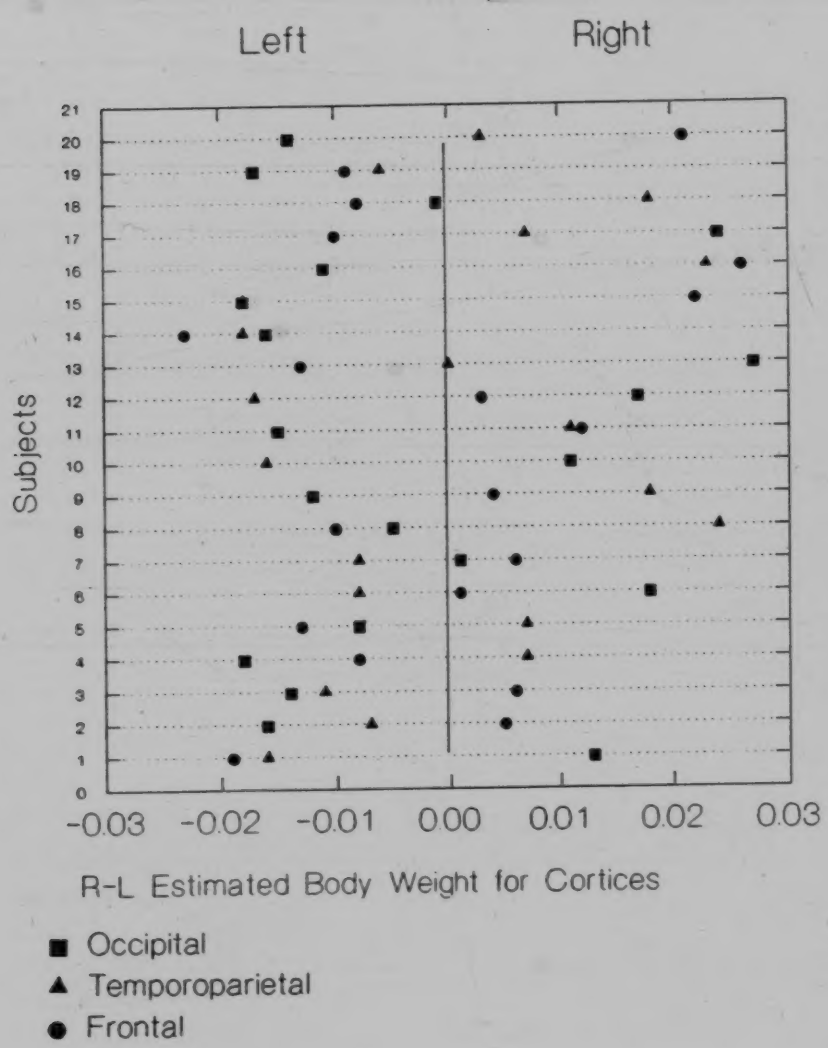
F-test comparisons of fluctuating asymmetry variances between cortical areas for B6D2F$_2$ and BALB/cWah1 fetuses.

Strain	Comparison	F	p
B6D2F$_2$			
	frontal vs. temporoparietal	$F_{(11,11)}=1.5$	>.01
	frontal vs. occipital	$F_{(11,11)}=2.57$	>.01
	temporoparietal vs. occipital	$F_{(11,11)}=1.71$	>.01
BALB/cWah1			
	frontal vs. temporoparietal	$F_{(7,7)}=3.23$	>.01
	frontal vs. occipital	$F_{(7,7)}=3.23$	>.01
	temporoparietal vs. occipital	$F_{(7,7)}=1.0$	>.01

some degree of asymmetry when compared to its homologue. In addition, the data were inspected for any evidence of a hemispheric growth advantage within an individual. On the horizontal lines in Figure 24 are the right-left differences for each cortex within a subject. These differences are rarely weighted to one side in any of the fetuses. For example, in most subjects a rightward growth advantage exhibited in one cortical area was followed by a leftward advantage in another cortex.

During the analysis of fluctuating asymmetry, FA appeared to be more pronounced in the frontal cortex than the temporoparietal or occipital cortices. This is apparent in the plot of R-L differences in estimated body weight, collapsed across strains

Figure 24. A scatterplot of the difference between right and left (R-L) estimated body weight within the frontal, temporoparietal and occipital cortices, for all subjects of Experiment 2. In this figure all points to the left of the scatterplot are indicative of greater left hemisphere growth and the opposite holds for points to the right of the scatterplot.



(see Figure 25). Had there been a concomitant increase in the amount of FA in the temporoparietal cortex then this result could be extrapolated to suggest a rostro-caudal asymmetry gradient. However, there was no indication of asymmetry being greater in the temporoparietal or occipital cortices. This would suggest that increasing the sample size might result in R-L distributions for the frontal cortex that more closely approximate a normal curve.

The FA analysis also pointed to a possible strain difference in the amount of cortical right-left asymmetry. The histograms of R-L estimated body weight, collapsed across cortices (see Figure 26), showed that the BALB/cWah1 fetuses exhibited larger right-left differences. Support for the possibility of increased asymmetry being present in the BALB/cWah1 fetuses is provided by Experiment 1, where strain differences in FA also approached significance. The lack of significant strain differences at the .01 criterion level in both Experiments 1 and 2 may be due to the relatively small sample of inbred fetuses. However, in conjunction, these results would support the anticipated result that CC axon growth for inbred fetuses is somewhat less symmetrical.

One of the drawbacks to the present experiment was the inability to investigate axon growth after the CC crossed midline. Since DiI had been injected in both hemispheres it was not possible to differentiate ipsi- and contralateral axon growth due to the fluorescence for each hemisphere being the same. Using a stereotaxic provided accurate measurements for a single pipette. However, to double label a brain successfully would require that two pipettes be used. This method was attempted, but changing the pipette made it extremely difficult to reestablish the settings used for the first set of

Figure 25. A density plot of R-L estimated body weight for each cortical region collapsed across subjects. This figure illustrates that, despite the greater spread of R-L differences in the frontal cortex, the majority of R-L differences are clustered close to 0.0 for all three cortical areas.

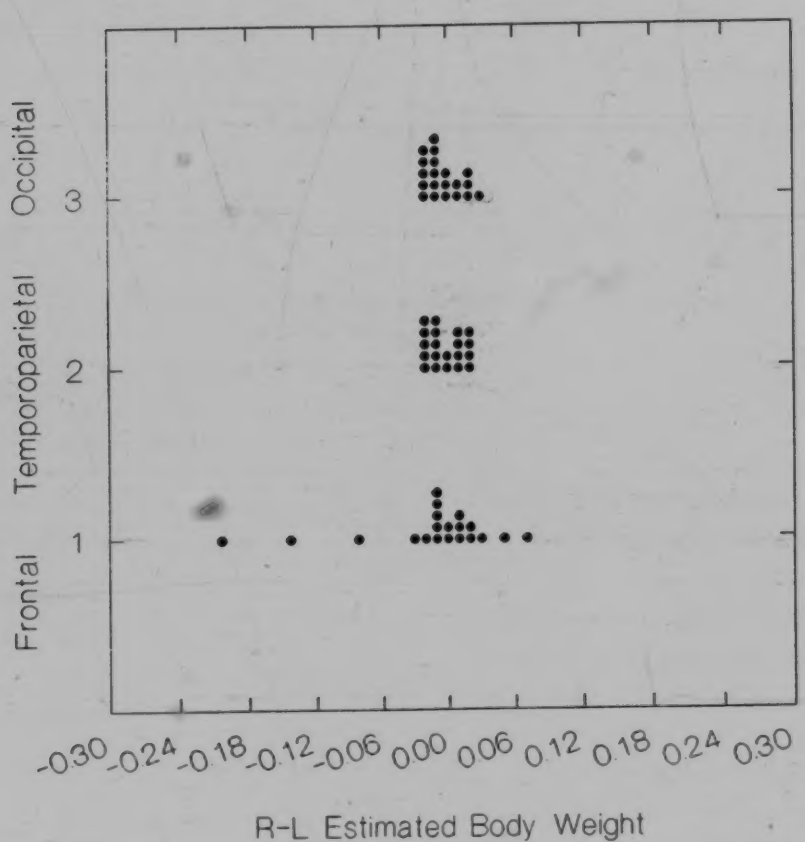
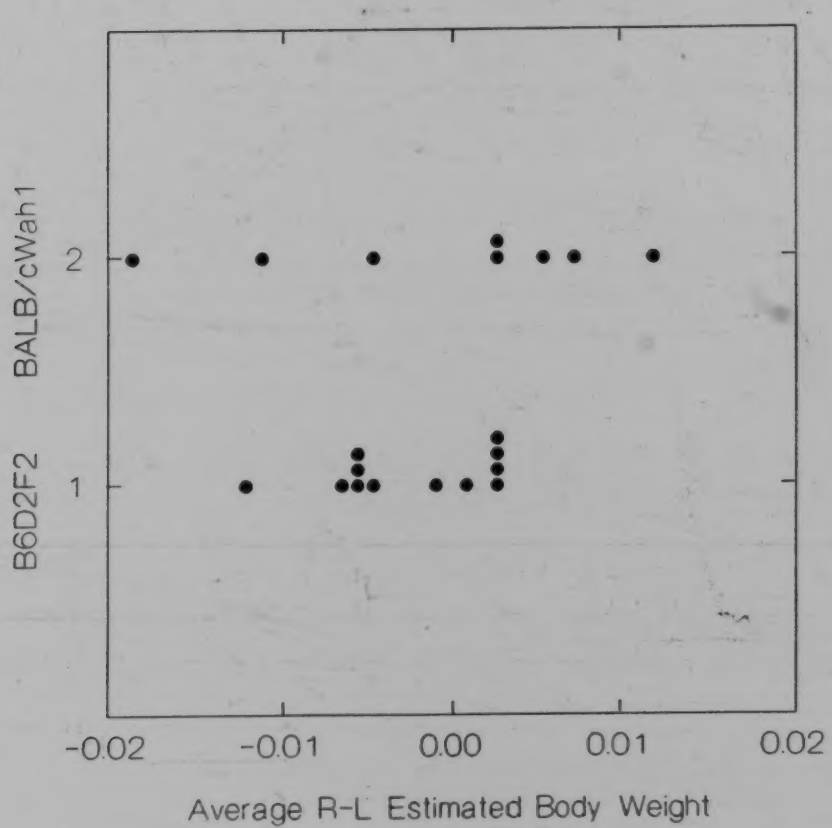


Figure 26. A density plot comparing the average R-L difference in body weight, collapsed across cortical areas, in the B6D2F$_2$ and BALB/cWah1 fetuses. This figure shows the greater variance of R-L for the inbred fetuses.



injections. The resulting difficulty in maintaining symmetry in the amount and location of dye injections precluded the use of double labelling for this experiment, because symmetry of dye injection sites was considered vital if valid conclusions were to be drawn about the symmetry of cortical growth.

In Experiment 2 ISMB was used to measure the distance the axons had grown. This measurement parameter was used first in the paper of Ozaki and Wahlsten (1992). Comparisons between the distances measured in the present study and distances predicted using the regression equations constructed by Ozaki and Wahlsten as shown in Figures 12, 13 and 14 indicated that the latter found axons had grown farther in fetuses of the same body weight. There are a number of possible reasons for Experiment 2 to have more conservative results. Ozaki and Wahlsten were measuring the distance from the insertion site to the growth cones, whereas the present study measured only the distance to the end of the main bundle of CC axons. Also, Ozaki and Wahlsten inserted dye crystals which may have labelled a greater amount of tissue than the discrete dye injections utilized in the present study. If the amount axon growth was variable within a cortical area the increased amount of labelling offered by a crystal would have allowed Ozaki and Wahlsten to accurately measure the full extent of growth within that cortex. In contrast, the solution provided very discrete amounts of labelling and may not have provided enough dye to completely determine the amount of axon growth in a cortex. That is, the fact that Ozaki and Wahlsten labelled more tissue may have enabled them to measure the fullest extent of CC growth exhibited by a cortical region.

EXPERIMENT 3 - EYE DEVELOPMENT

Introduction

Experiment 3 was undertaken primarily to investigate the hypothesis that an overall asymmetry gradient may exist throughout the developing fetus. It is possible that the same developmental phenomenon that is responsible for asymmetry in the external morphology may also influence the developing symmetry of the central nervous system. Additionally, asymmetry in neural development may be small. Therefore, studies of prenatal ontogeny could be aided by studying the external morphology of the fetus (Wahlsten, 1981). The eye was considered a useful biological trait to study because eyelid growth, fusion and reopening are common to all mammals (Harris & McLeod, 1982). In addition, the sequence of eyelid growth is sensitive to a variety of environmental and genetic perturbations (Tyndall & Sulik, 1991) and may be a good indicator of the quality of embryonic development. To test this hypothesis the schedule of eye closure was measured in a standard control strain and two inbred strains.

Methods and Materials

The subjects for this experiment were 41 B6D2F_2, 44 C57BL/6J and 14 DBA/2J fetuses. Fetal mice of gestational age E15.0 to E17.5 were obtained by the extraction method described previously. However, instead of intracardiac perfusion the fetuses were immersed in Bouin-Dubosq fixative immediately after extraction, and after 48 hours they were reweighed and placed in 70% ethanol. The fetuses became dehydrated while in ethanol (body weight decreased approximately 0.2 g) but stabilized after 7 days.

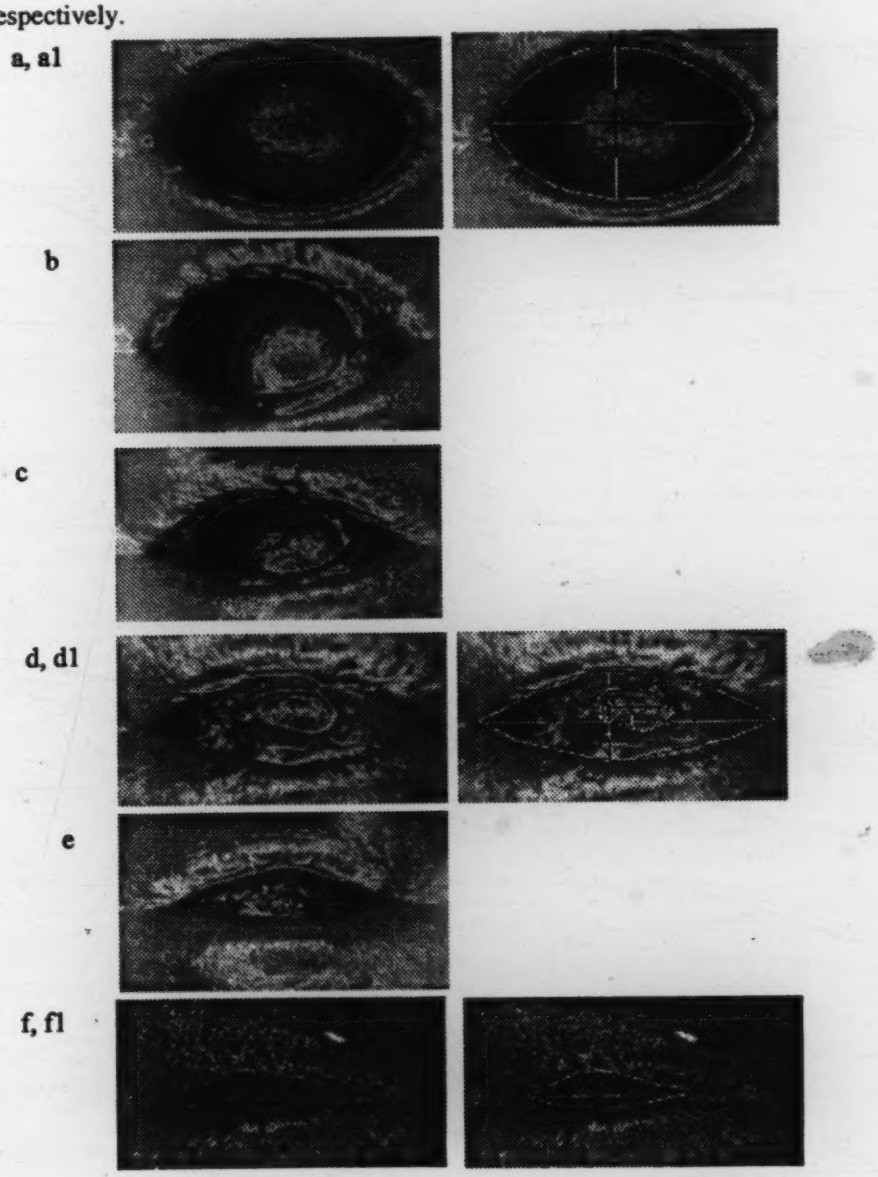
The fixed fetuses were imaged using Jandel Video Analysis Software (JAVA) in combination with a Wild M3Z photographic microscope and CCD camera. The

photographic microscope and camera were used to obtain an image of the eye, the picture was then captured and stored using JAVA. To avoid experimenter bias the side that was imaged first was chosen randomly and labeled as side 1, then the fetus was turned over and the opposite side was imaged and recorded as side 2. A master list with which side corresponded to 1 and 2 was kept separately. Afterwards, the stored JAVA images were recalled and the height, width, circumference and area of the eyes were measured (in mm and mm^2; see Figure 27).

Eye height was defined as the vertical distance of the exposed corneal epithelium. Before the eyelids begin to form, the cornea and the eyelids were clearly demarcated by a ring of space between them and the lens was visible as a white disc in the centre of the eye. At this point eye height equaled eyelid height. When the eyes began to close there was an increase in epithelial cell activity at the peripheral margin of the eyelids (Kaufman, 1992) and as development proceeded this cellular activity increased, thereby decreasing the area of exposed cornea. As can be seen in Figure 27 the cellular exuberance occurred in advance of the eyelids fusing. In some older fetuses the corneal epithelium had been completely covered by the encroaching cell layer even though the eyelids had not yet fused. In these cases the eye measurements recorded how much, if any, of the cornea was exposed, while the second measurement followed the progress of the eyelid closing.

Similarly, the eye width is the horizontal distance of the exposed cornea. Whereas the eye shows no sign of the eyelid cell layer developing, width is the distance from the inner canthus to the outer canthus and eye width equals eyelid width. The inner canthus is

Figure 27. Photographs of the eyes of B6D2F$_2$ fetuses showing eye closure at a variety of stages. Photographs a1, d1, f1 also illustrate the parameters used for measuring the height of the eye and eyelid, when the cornea is completely exposed, partly exposed and covered, respectively.



identifiable as a dip in the circumference of the open eye (Tyndall & Sulik, 1991). When eyelid cells begin to develop, width, like height, is a measurement only of the exposed cornea.

The circumference of the eye follows the border of the cornea and early is equal to the circumference of the eyelids, prior to eyelid formation. When the eyelid forms, the circumference of the cornea is still measurable inside the ring of encroaching cells.

Results

Reliability was tested using 20 B6D2F_2 fetuses. Right and left eye height and area were remeasured and a Pearson correlation was calculated using both sets of measurements. The r values for all four variables was 0.9 or greater, indicating a high intra-observer reliability.

Comparison plots made of all variables indicated that not all were equally useful. Eye width did not accurately reflect development because the width changes little and is easily measurable along the point of fusion even after the eye has almost completely closed (Kaufman, 1992). Furthermore, the height, area and circumference of the cornea (i.e. eye) followed a very similar pattern of development and were highly intercorrelated. The multiple R^2 of a regression equation predicting weight using height, area and circumference was 0.671. However, regressing only height on weight provided an R^2 of 0.667. Therefore, eye height was preferentially used because it appeared to be the most sensitive measurement. In addition, 4 cases were removed as outliers, three C57BL/6Js and one B6D2F_2, because they showed a degree of asymmetry beyond what may have been expected within normal development. The C57BL/6J strain is known to have a

genetic predilection for a right eye defect that occurs in approximately 10% of animals (Tyndall & Sulik, 1991). A subject was considered an outlier if the amount of asymmetry was greater than three standard deviations from the mean. Given the extent of malformation in these animals and difference in degree of closure between the two eyes (see Figure 28), it was concluded that the asymmetry seen was not representative of the populational asymmetry under investigation.

Using all the data points for each animal within a strain illustrated that the development of the eyes was nonlinear. Height increased as the fetuses grew and the external embryonic eye appeared (peaking at approximately 0.25 to 0.3 g) and then dropped off rapidly as the lid formed and the eye began to close, eventually reaching an asymptote when the eyelids were fully fused (see Figure 29). Therefore, the data were truncated in order to isolate the linear segment of the distribution, since the linear portion of the curve represents the time when development is proceeding most rapidly and therefore may be the most sensitive to differences in right and left. A datum was removed only if eye height equalled eyelid height. This method of truncation ensured that only cases where the eyes were still closing were considered. The fetus in question was only removed from the data set if both eyes fulfilled the above requirements. In this way any fetus that displayed an asymmetrical pattern of eyelid closure was retained. This produced plots for all three strains that could be more accurately characterized using linear regression (r^2=0.667) because adding a quadratic term to the regression equation failed to improve goodness of fit (R^2=0.667) (e.g. see Figure 30). However, one disadvantage that became immediately apparent after truncating the data was the

Figure 28. A scatterplot of right minus left eye height versus weight in (a) B6D2F$_2$ and (b) C57BL/6J fetuses. The subjects at the extremes of the distribution (→), one B6D2F$_2$ and three C57BL/6J subjects, were removed from the analysis.

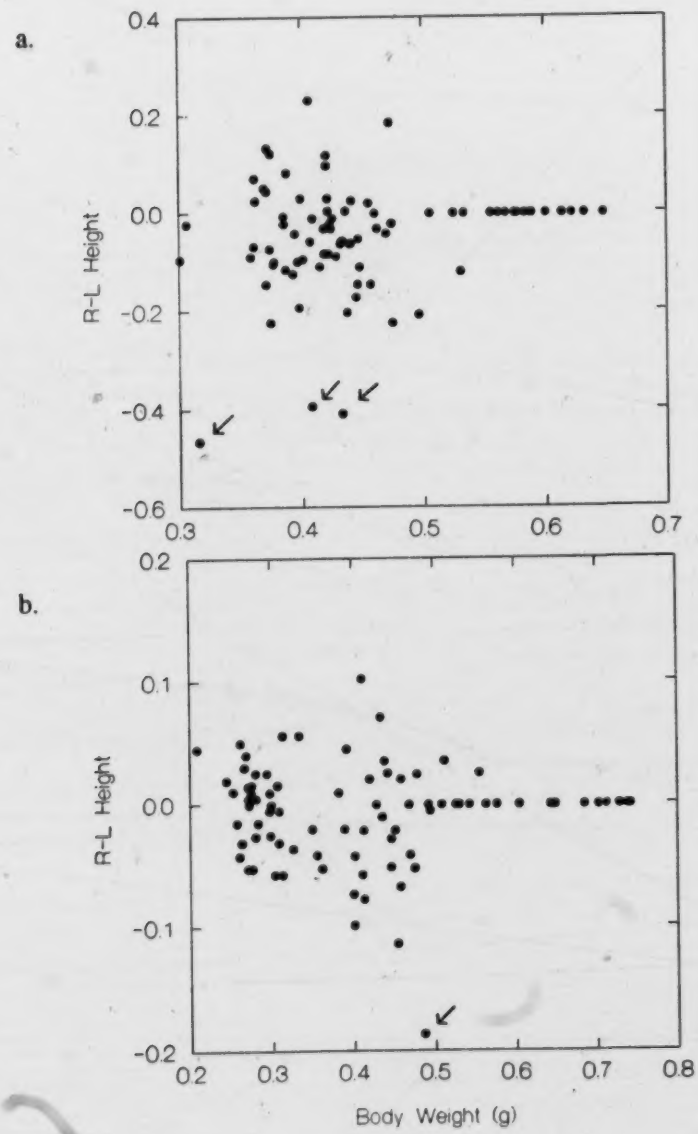


Figure 29. A scatterplot of right and left eye height versus weight in B6D2F$_2$ fetuses, illustrating the nonlinearity of eye closure. Note: the lines of best fit applied to the scatterplot were obtained using the "lowess" smoothing function (tension=0.5) in SYSTAT.

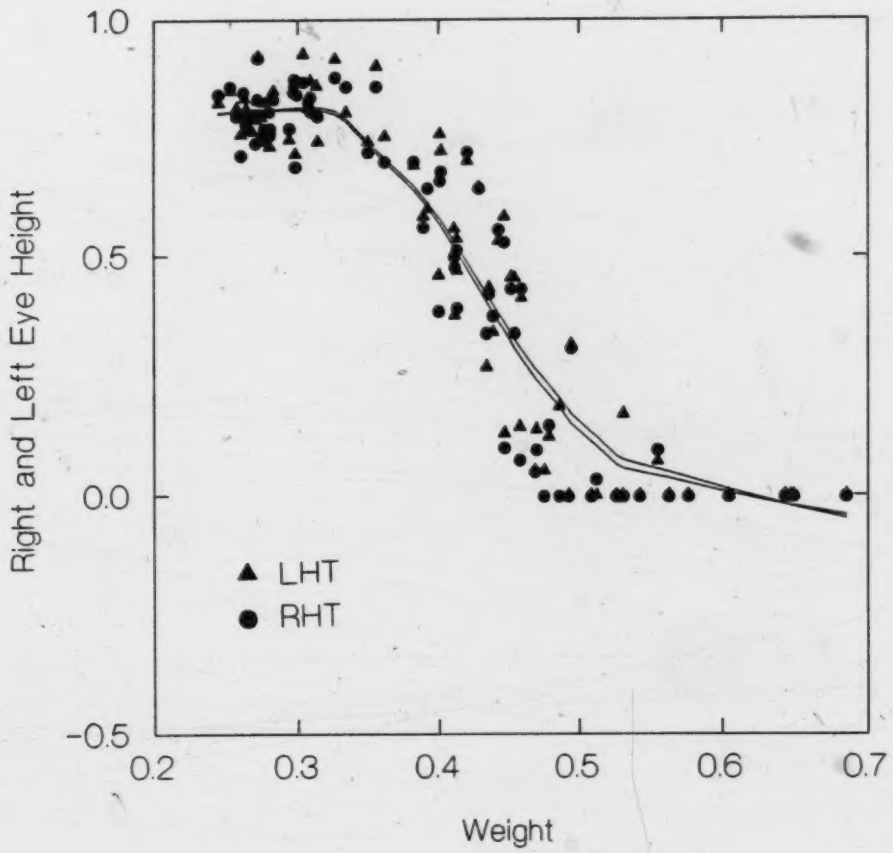
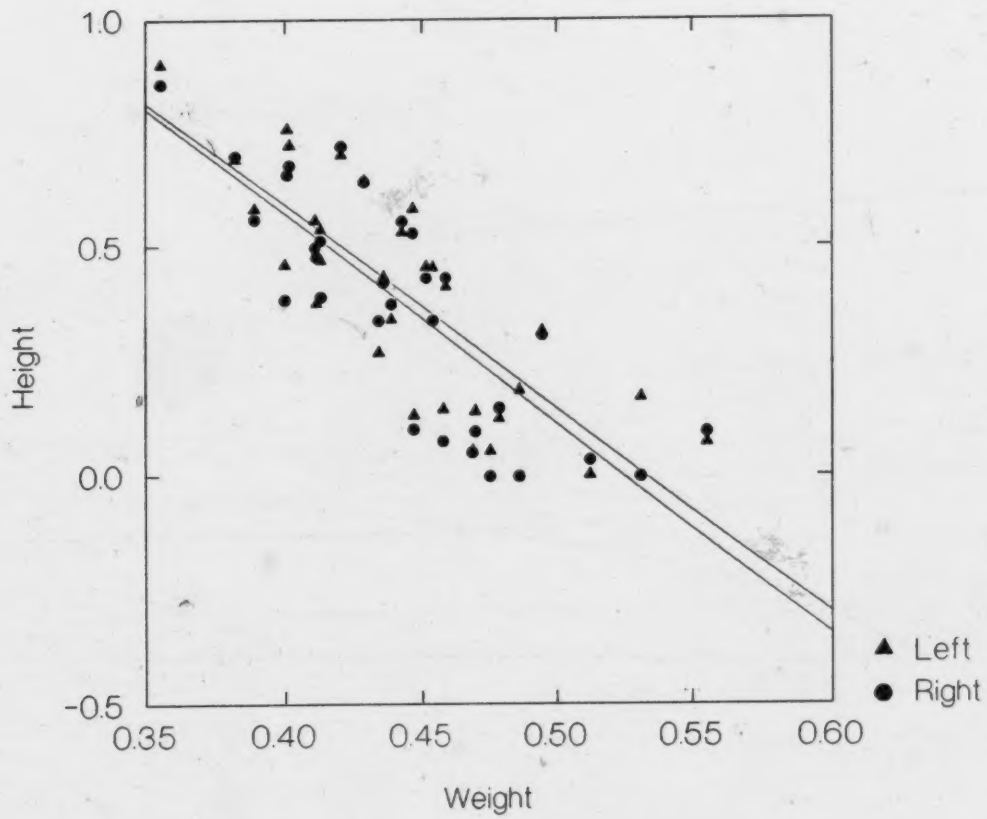


Figure 30. A scatterplot of right and left eye height as a function of weight, using the truncated B6D2F$_2$ data set. This example shows that the measurement of eye closure can be characterized with linear regression when the ends of the distribution are removed.



reduction in sample size, especially for the DBA/2J group.

The estimated body weight (EBW) equations used to predict the amount of development for each side were derived specifically from each strain, rather than using the equation from the B6D2F_2 group as a standard. This was done because the experiment lacked a standard equation, and therefore the results may have been biased. For the B6D2F_2 fetuses (EBW=0.5-0.147(HT)) multiple t-tests performed on the EBW for each side indicated that the amount of development did not differ significantly between the right eye height and left eye height ($t=0.85$, $p>.01$). Within the inbred strains the C57BL/6J fetuses (EBW=.47-.117(HT)) did not show a difference between the right and left eye closing ($t=2.6$, $p<.01$); and the DBA/2J fetuses (EBW=.569-.167(HT)) also did not show any difference between which eye closed first ($t=0.681$, $p>.01$). The pooled difference between the hybrid and inbred strains also failed to reach significance ($t=1.328$, $p>.01$).

As above, the possibility of fluctuating asymmetry (FA) was investigated. The first step was to analyze plots of the difference in EBW between sides versus body weight for all three strains to ensure that no relationship existed between symmetry and growth. Plots for all three strains confirmed that scaling was not necessary (see Figures 31, 32, 33). Histograms of R-L were constructed and for each strain the resulting distributions were reasonably close to normal (see Figures 34, 35, 36) and to ensure the means were centred around zero, t-tests were used to compare the distribution mean to the expected population mean. The results for the B26D2F_2 ($t=-0.431$, $p>.01$), C57BL/6J ($t=-0.534$, $p>.01$) and DBA/2J ($t=1.328$, $p>.01$) fetuses all failed to reach significance.

Figure 31. A scatterplot of right estimated body weight minus left estimated body weight (R-L) versus weight for B6D2F$_2$ fetuses, showing that no relationship existed between asymmetry of eye closure and fetal growth.

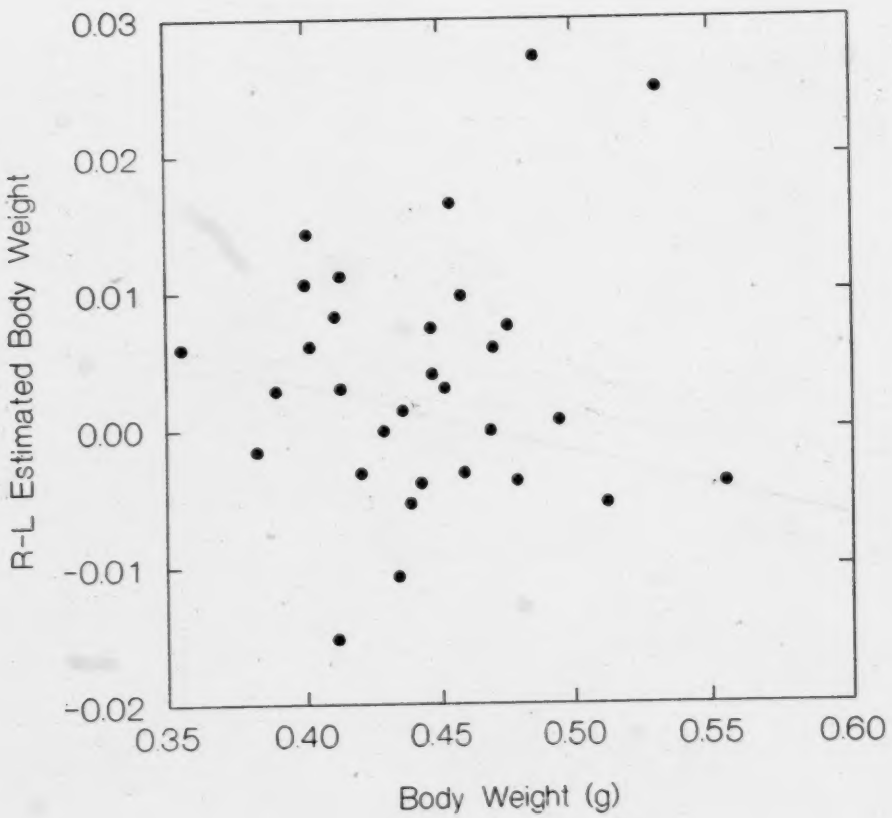


Figure 32. A scatterplot of right estimated body weight minus left estimated body weight (R-L) versus weight for C57BL/6J fetuses, showing that no relationship existed between asymmetry of eye closure and fetal growth.

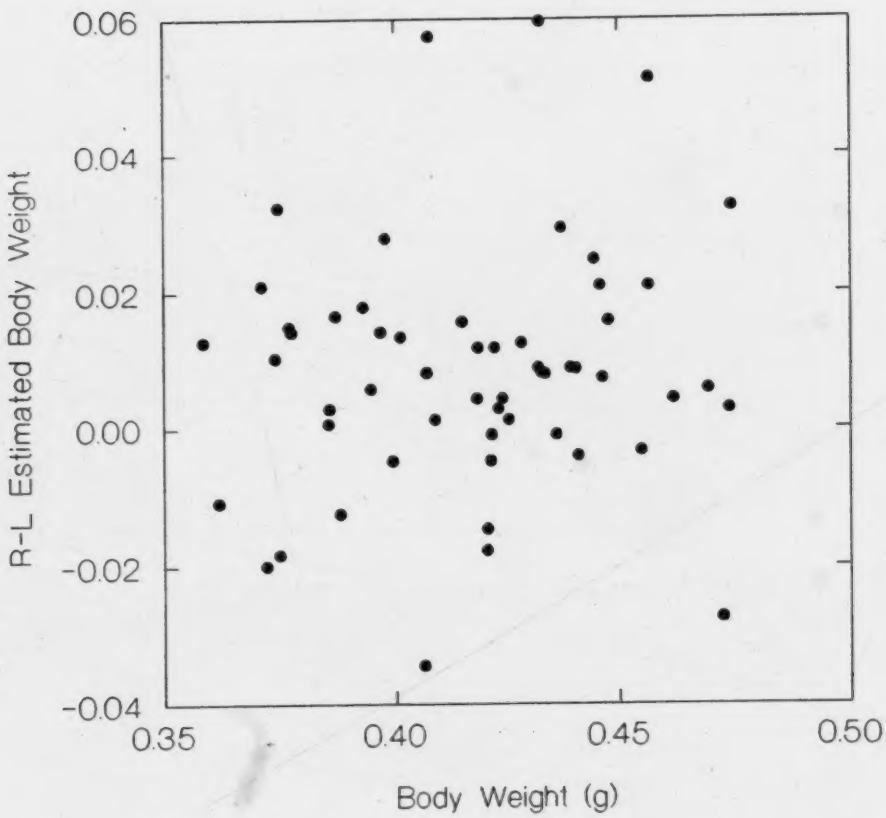


Figure 33. A scatterplot of right estimated body weight minus left estimated body weight (R-L) versus weight for DBA/2J fetuses, showing that no relationship existed between asymmetry of eye closure and fetal growth.

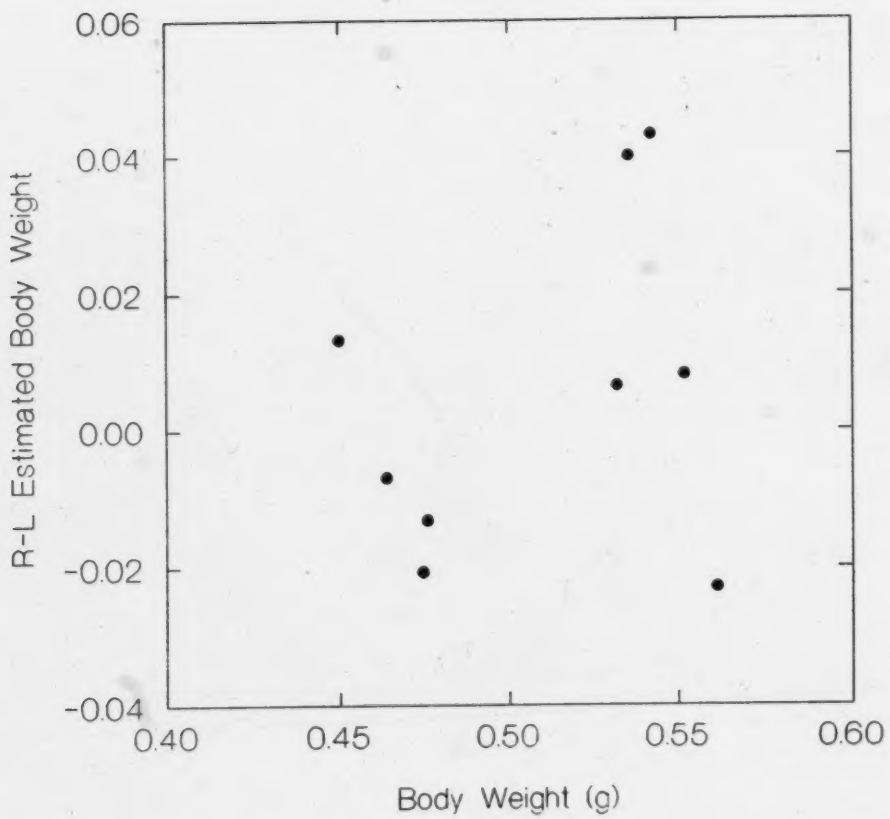


Figure 34. A histogram of the difference between right and left estimated body weight (R-L) for B6D2F$_2$ fetuses showing a normal distribution around a mean of zero, indicative of fluctuating asymmetry.

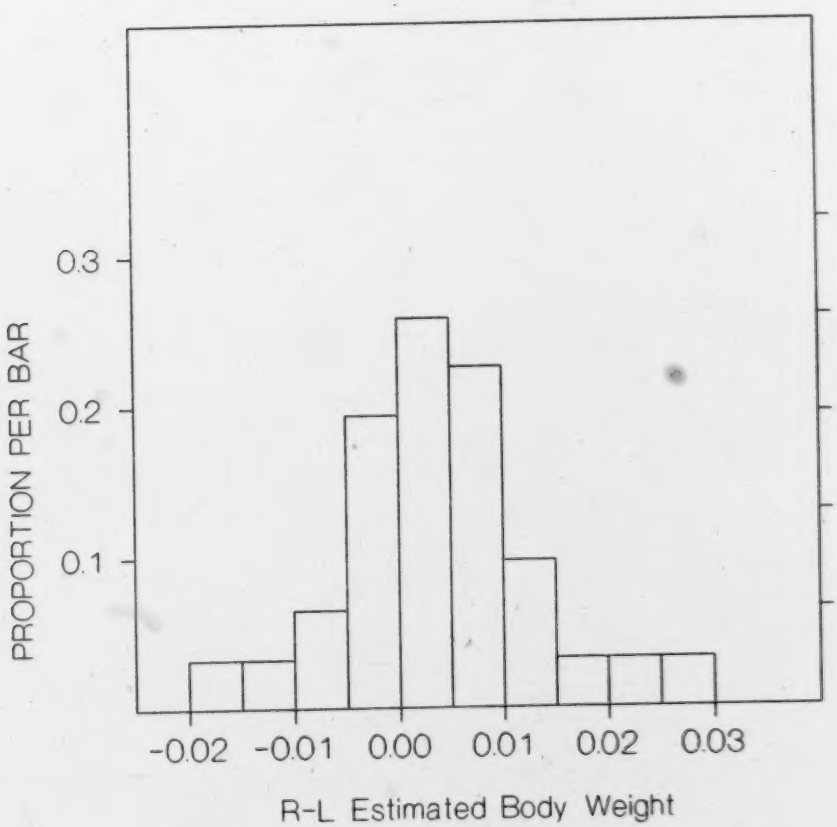


Figure 35. A histogram of the difference between right and left estimated body weight (R-L) for C57BL/6J fetuses showing a normal distribution around a mean of zero, indicative of fluctuating asymmetry.

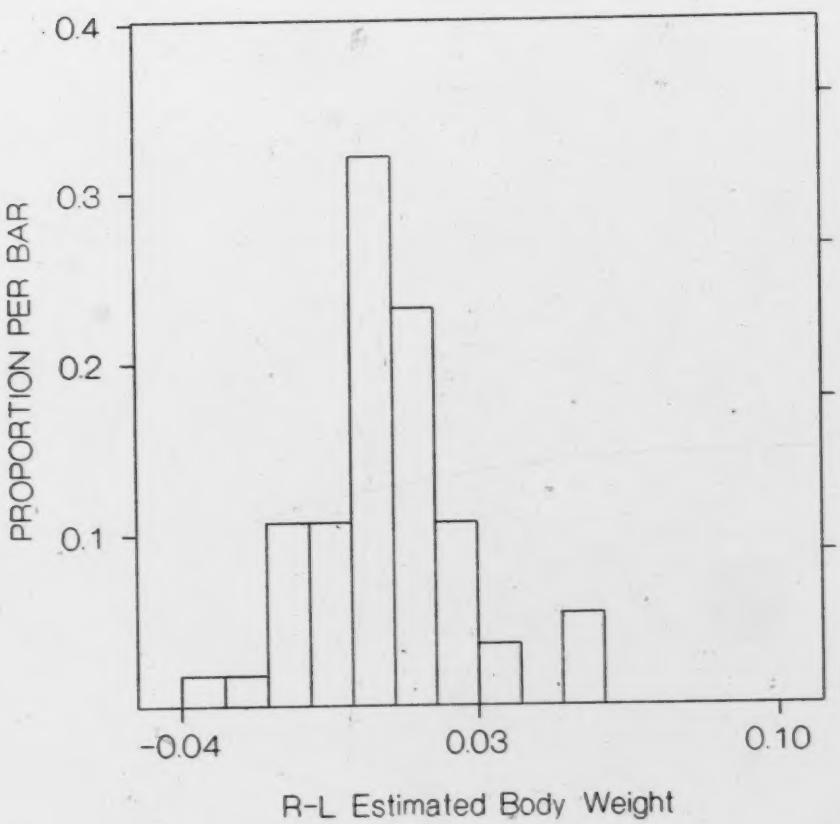
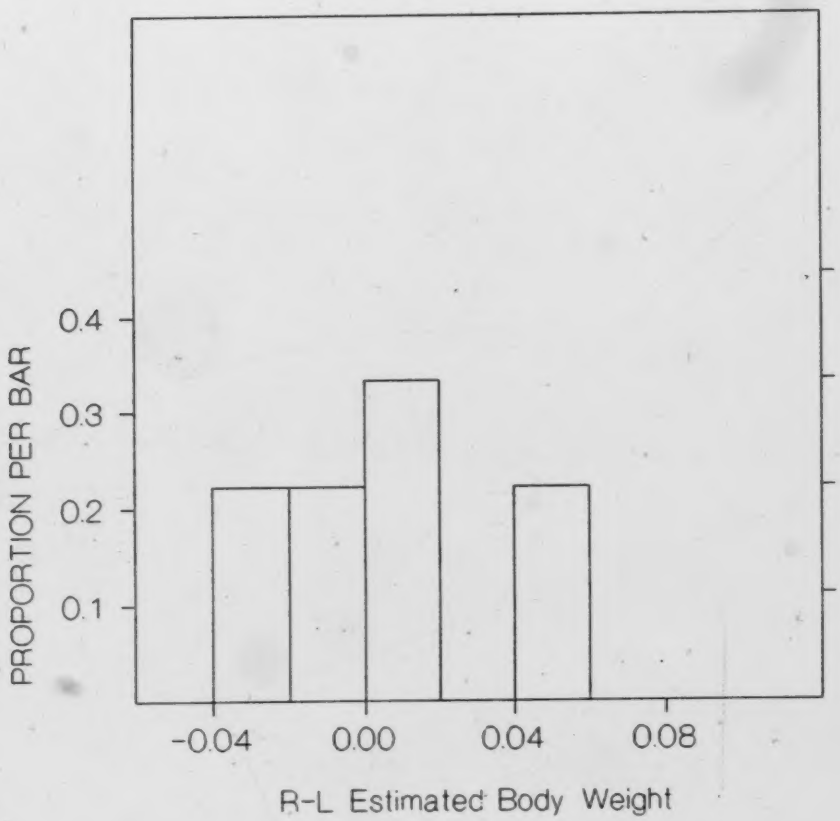


Figure 36. A histogram of the difference between right and left estimated body weight (R-L) for DBA/2J fetuses showing a normal distribution around a mean of zero, indicative of fluctuating asymmetry.



As in earlier experiments, a mixed model ANOVA within Sides and Between Strains was utilized to test for the presence of directional asymmetry (DA)(see Table 9). Neither the result for Strain ($F_{(2,93)}$=1.895, p>.01), Side ($F_{(1,93)}$=4.288, p>.01), nor the interaction between Side and Strain ($F_{(2,93)}$=0.039, p>.01) was significant, indicating the absence of DA.

Fluctuating asymmetry for each strain was computed using the index FA=var(R-L). F-tests were used to compare FA between samples (see Table 10)(Kirk, 1982). The difference between the B6D2F_2 and C57BL/6J mice was significant ($F_{(55,30)}$=3.75, p<.01), as was the difference between the B6D2F_2 and DBA/2J fetuses ($F_{(8,30)}$=6.75, p<.01), whereas the difference in FA between the two inbred strains was not significant ($F_{(8,55)}$=1.8, p>.01).

Table 9

Results for between Strains and within Side ANOVA of eye height for Experiment 3.

Source	Sum of Squares	df	Mean Square	F	p
Strain	0.346	2	0.173	1.895	>.01
Error	8.491	93	0.091		
Side	0.027	1	0.027	4.288	>.01
Strain x Side	0.0046	2	0.0023	0.039	>.01
Error	0.578	93	0.006		

Table 10

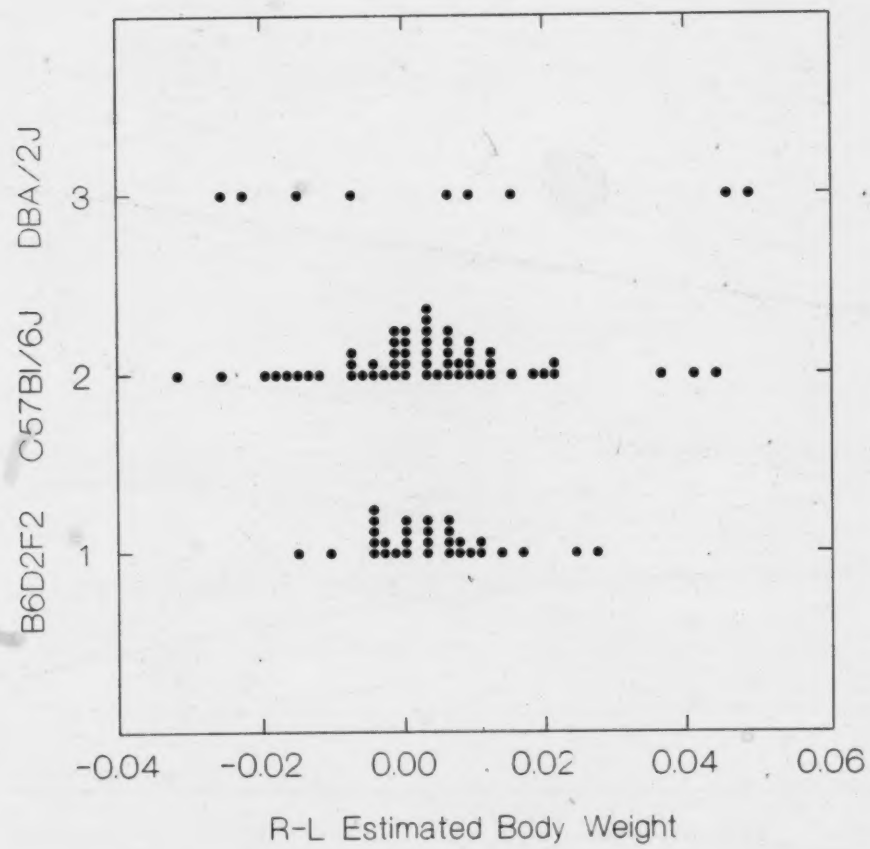
Mean difference between right and left eye height, variance of eye height difference and variance of estimated body weight difference, obtained in fluctuating asymmetry analysis of Experiment 3 for B6D2F$_2$, C57BL/6J and DBA/2J fetuses.

Strain	Mean Height	Height Variance	EBW Variance
B6D2F$_2$	-0.026	0.004	0.00008
C57BL/6J	-0.030	0.015	0.0002
DBA/2J	-0.037	0.027	0.001

Discussion

The results from the present study suggest that there is no large directional asymmetry in the timing of the right and left eyes closing. This is in agreement with Juriloff (1987) who, while studying a cortisone treatment for lidgap mutation, judged eye closure to be asymmetrical in less than 10% of cases. In addition, the lack of directional asymmetry is consistent with the above experiments. However, although the difference between sides showed a characteristic lack of directional asymmetry, the amount of fluctuating asymmetry did differ significantly between strains. It appeared that the hybrid B6D2F$_2$ strain showed smaller right-left differences than either of the inbred strains (see Figure 37), and the greater amount of FA in the C57BL/6J and DBA/2J fetuses was confirmed with the significant results obtained between the hybrid and the two inbred strains. An inverse relationship has been posited between the amount of FA and the fitness of an organism (Emlen, Freeman & Graham, 1993; Palmer & Strobeck, 1986). It

Figure 37. A density plot of R-L differences in estimated body weight for B6D2F$_2$, C57BL/6J, and DBA/2J fetuses, indicating that the inbred fetuses showed greater differences between right and left eye closure when compared to the hybrid fetuses.



has been established that inbred fetuses develop more slowly than hybrid B6D2F$_2$ fetuses (Wahlsten & Wainwright, 1977) and in the present experiment these strains also exhibited a greater amount of FA. This result supports the contention that the degree of genetic homozygosity may be one of the factors in determining the severity of FA (Clarke, 1993; Phelan & Austad, 1994).

EXPERIMENT 4 - PAW DEVELOPMENT

Introduction

This experiment was undertaken to investigate further the second hypothesis that an overall asymmetry gradient may be present in the developing fetus. The fusing of the paw digits occurs at approximately the same prenatal stage as eye closing (Maconnachie, 1979). Previous work done in this laboratory had indicated that within the developing organism the paw was also subject to asymmetry (Stelmaschuk, personal communication). As such, the degree of symmetry was measured in prenatal paw development.

Methods and Materials

The subjects were 36 B6D2F$_2$ and 37 BALB/cWah1 fetuses obtained for Experiment 2 and perfused using the methodology described above.

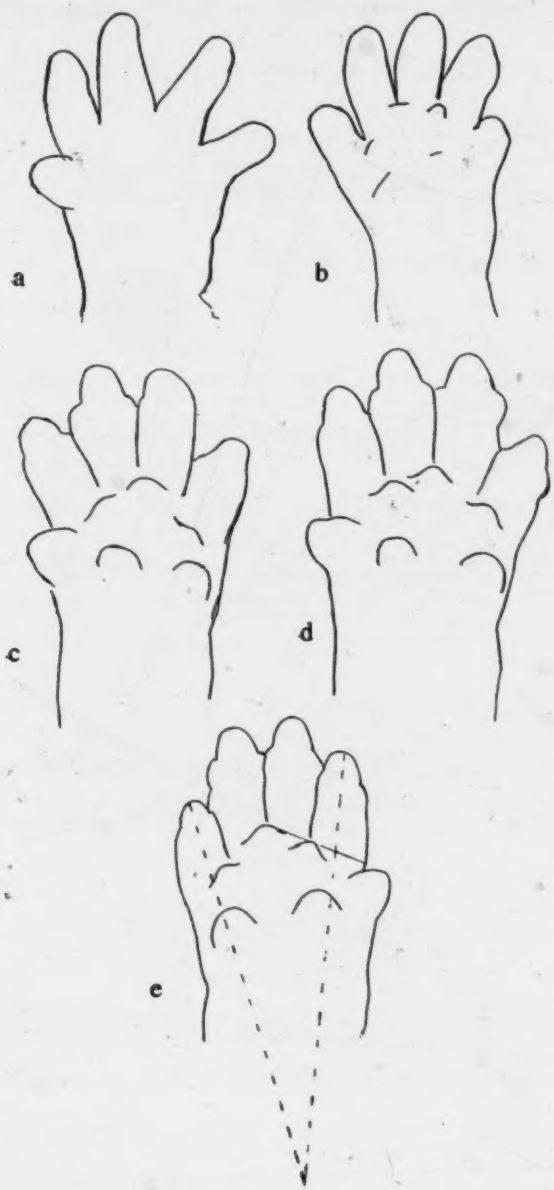
The present study utilized only the forelimbs cut off at a point near the elbow joint. The limbs were then placed in a concave microscope slide so they remained in fixative while being viewed, thus preventing damage due to drying. The paws were viewed using a 10X eyepiece in a Dialux microscope at 4X magnification with a Leitz-Weitzlar tracing device. The tracings followed all four digits and the thumb, and included

all three paw pads (see Figure 38). Some paws were not traceable due to damage incurred while being taped down during the perfusion process.

Using SigmaScan (Jandel Scientific) interdigit angle and digit length were measured. Prior to being measured the tracings were prepared by drawing a straight line that touched the lateral portion of each interdigit paw pad. The midpoint of each digit at the first knuckle was then determined and a straight line was drawn from the tip of the digit, through this midpoint, perpendicular to the digit base line. This line was extended far enough that it crossed the same lines drawn for each other digit. This allows the interdigit angle for any two digits to be calculated. Digit length was calculated by measuring the perpendicular line from the digit base to the digit tip. In the present study all six possible interdigit angles were measured as was the length of each digit (see Figure 38).

Analysis of the data was done using multiple regression. Previous work in this laboratory had determined that the paw perimeter and the widest interdigit angle ($\angle 3$), in that order, best predicted the weight of the fetus (Stelmaschuk, personal communication). The widest interdigit angle, angle three, was the angle between the first and fourth digits. This angle was thought to best represent the decreasing "spread" of the paw as all the digits began to fuse together. However, perimeter could not be used to predict body weight in the present sample because the measurements were not valid. Stelmaschuk had used fetuses that were fixed by immersion. In contrast, the present study used fetuses that were fixed by intracardiac perfusion. Perfusion requires that the heart be exposed and to accomplish this the fetus must be secured in place by being taped across the paws. It was

Figure 38. Diagram of paw tracings for $B6D2F_2$ fetuses showing the course of prenatal paw development. On the final diagram (e) the parameter for measuring the interdigit angle ($\angle 3$) is illustrated.



during this process that the damage to the paws was incurred. As a result the regression equation for the widest interdigit angle ($\angle 3$) was used to determine a predicted body weight. The estimated body weight (EBW) for each side was then compared to the predicted body weight for the opposite side. Multiple pairwise t-tests were used to determine if the EBW for each side differed, and to test if individual side differences departed from the expected population difference (0).

Results

In order to determine if the current data were applicable to the equation derived by Stelmaschuk, a comparison was made between the Stelmaschuk equation and the equation derived from the average of the right and left sides of the $B6D2F_2$ sample. The slopes of Stelmaschuk and the present experiment did not differ significantly ($t=-2.12$, $p>.01$) so the former equation was used ($\ln EBW=3.329-1.09(\ln\angle 3)$).

As above, an EBW for each side was computed by inserting the actual measure for angle 3 for each subject into the Stelmaschuk equation. Using the log of angle 3 in the above equation produced the natural log (ln) of the estimated body weight. The result was subjected to a log transformation because the work of Stelmaschuk had identified the data as significantly nonlinear, and this nonlinearity could be eliminated by transforming the data. Using the hybrid fetuses paired t-tests on the EBW for each side indicated that the amount of paw development for each side was not significantly different ($t=0.652$, $p>.01$). The same comparison for the inbred fetuses was also not significant ($t=-0.872$, $p>.01$). Afterwards, the EBW needed to be compared to that from other experiments. To do this the earlier log transformation needed to be eliminated and this was accomplished

by computing the antilog of EBW. As has been done in earlier experiments, FA was also investigated. The first step was to plot R-L differences against body weight. No pattern emerged (see Figures 39, 40), so the data were not scaled. Histograms of R-L were approximately normal (see Figures 41, 42), but the plot of R-L for the BALB/cWah1 fetuses appeared to have a slight positive skew. Both distributions were tested against the expected population difference and the result for neither the $B6D2F_2$ ($t=0.681$, $p>.01$) nor the BALB/cWah1 ($t=-1.217$, $p>.01$) was significant. To test for the presence of DA an ANOVA was constructed using Strain as the between factor and Side as the within factor (see Table 11). Directional asymmetry was not apparent in the sample because neither Side ($F_{(1,81)}=1.33$, $p>.01$) nor the Strain by Side interaction ($F_{(1,81)}=0.11$, $p<.01$) was significant. The main effect of Strain was significant ($F_{(1,142)}=11.4$, $p<.01$), meaning that the amount of asymmetry appeared to be greater in the BALB/cWah1 fetuses. However, an F-test on the measure of FA obtained from each strain failed to reach significance at the .01 criterion, but was significant at the .05 level ($F_{(36,35)}=2.3$, $p=.028$) (see Table 13).

Discussion

The results from this experiment lend further support to the contention that directional asymmetry (DA) is primarily absent in the developing fetus. Differences between the sides are apparent, however they are not systematic. Of note in this experiment was that, similar to Experiment 4, the amount of FA was greater in the BALB/cWah1 fetuses, though the differences between hybrid and inbred fetuses were not as dramatic, as can be seen in Figure 39.

Figure 39. A scatterplot of right estimated body weight minus left estimated body weight (R-L) versus weight for B6D2F$_2$ fetuses, showing that no relationship existed between asymmetry and character size.

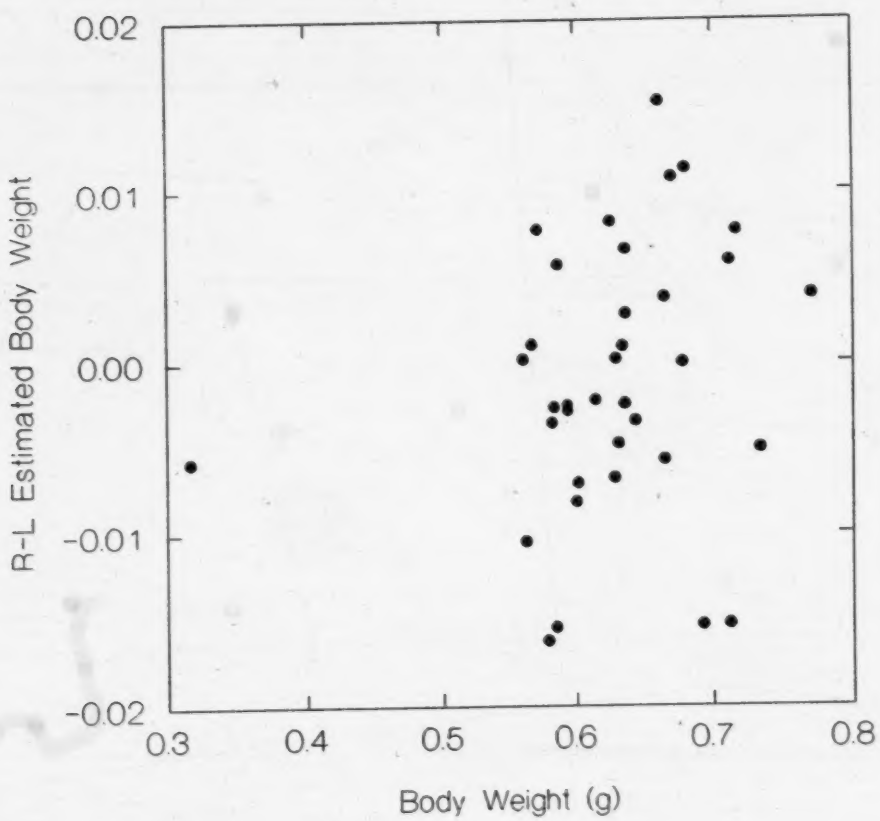


Figure 40. A scatterplot of right estimated body weight minus left estimated body weight (R-L) versus weight for BALB/cWah1 fetuses, showing that no relationship existed between asymmetry and character size.

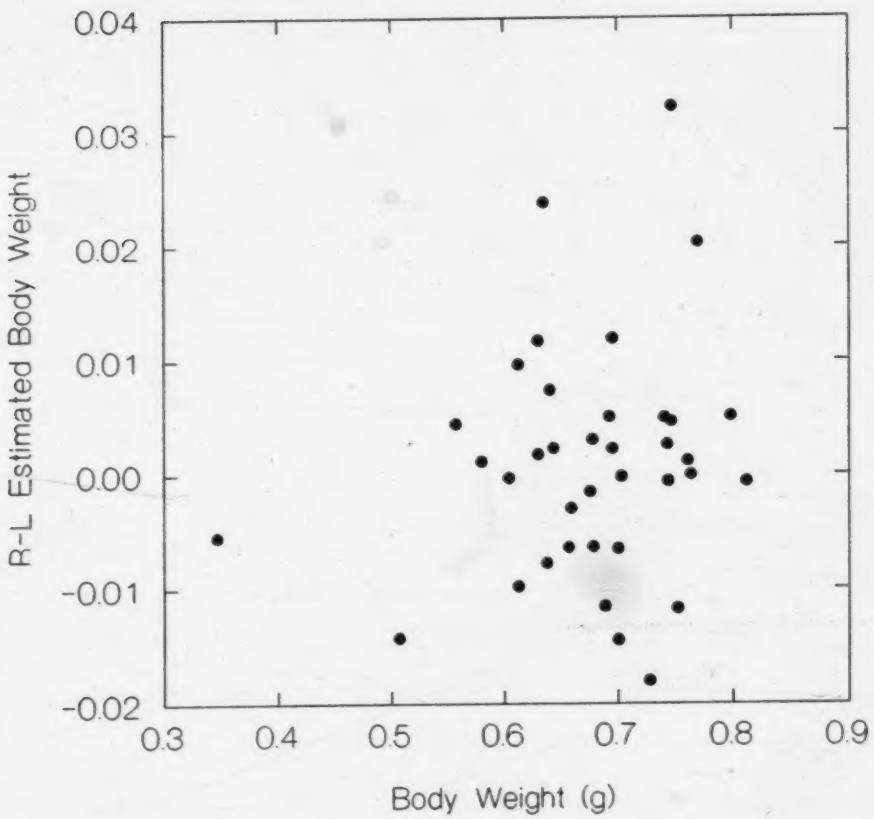


Figure 41. Histogram of the difference between right and left estimated body weight (R-L) for B6D2F$_2$ fetuses showing a normal distribution around a mean of zero, indicative of fluctuating asymmetry.

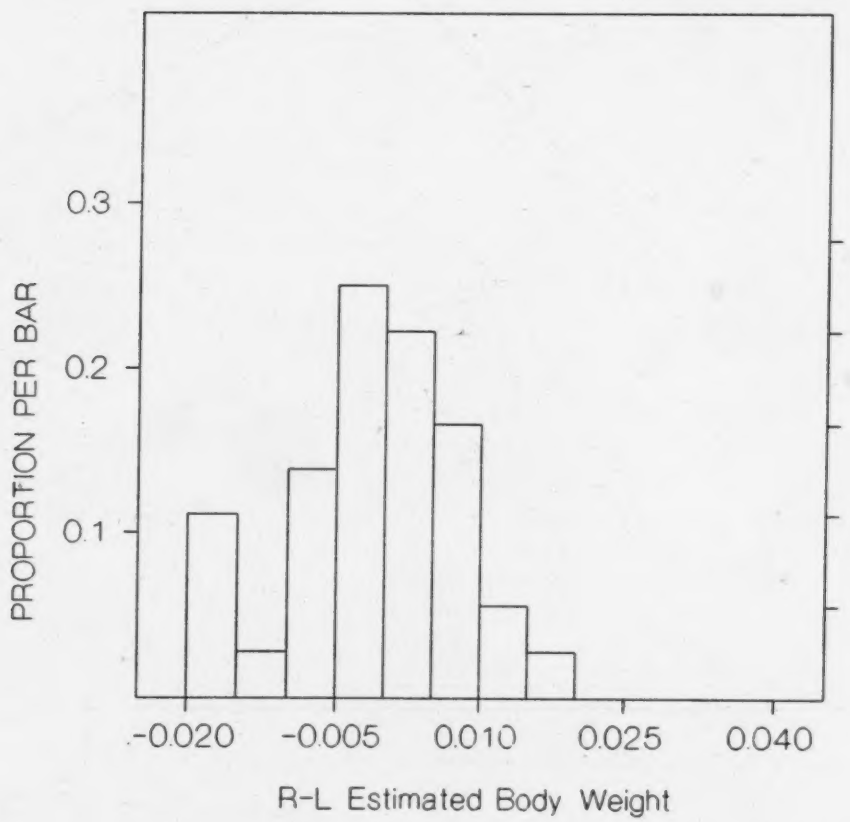


Figure 42. Histogram of the difference between right and left estimated body weight (R-L) for BALB/cWah1 fetuses showing a normal distribution around a mean of zero, indicative of fluctuating asymmetry.

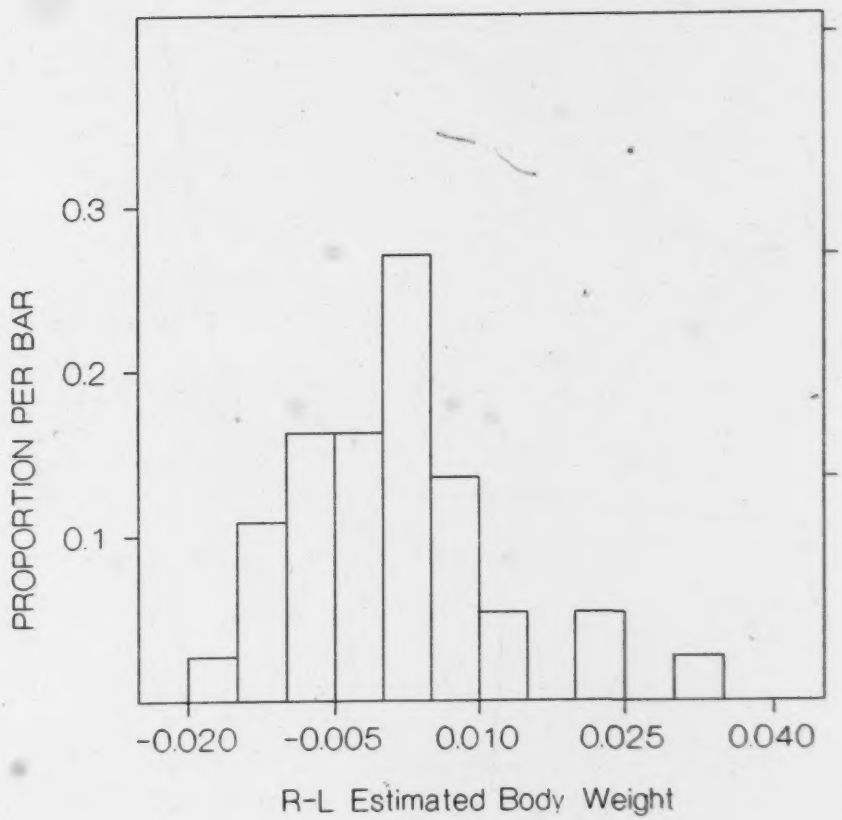


Table 11

Results from the between Strain, within Side ANOVA of interdigit angle for Experiment 4.

Source	Sum of Squares	df	Mean Square	F	p
Strain	0.567	1	0.567	11.395	<.01
Error	4.029	81	.0497		
Side	0.072	1	0.072	1.331	>.01
Side x Strain	0.006	1	0.006	0.111	>.01
Error	4.393	81	0.054		

Table 12

Mean difference between right and left interdigit angle, variance of interdigit angle difference and variance of estimated body weight difference obtained in fluctuating asymmetry analysis of Experiment 4, for B6D2F$_2$ and BALB/cWah1 fetuses.

Strain	Mean Angle Difference	Angle Variance	EBW Variance
B6D2F$_2$	0.029	0.0515	0.00006
BALB/cWah1	-0.043	0.0667	0.00011

The primary limitation of this experiment was the use of fetuses that had been fixed by perfusion because this method is more damaging to the fetus. Had the fetuses been fixed by the Bouin-Dubosq method, right-left differences in angle 3 may have been more apparent. In addition, the more powerful predictor of paw perimeter could have

been obtained from this sample. However, even if the preferred measurement circumstances had been available, it is doubtful whether any directional gradient in growth would have been revealed. There had been no trend in the prior investigations towards directional asymmetry and the results of the present experiment did not lend themselves towards indicating the presence of DA.

GENERAL DISCUSSION

Determination of a possible directional asymmetry in the growth of the corpus callosum and external morphological features was the focus of the first two hypotheses under investigation. The results from the present set of studies indicate that the fetuses of hybrid and inbred mouse strains fail to demonstrate any consistent directional asymmetry in their development. Instead, it appears that asymmetry in the developing mouse fetus is individual.

The focus of Experiments 1 and 2 was the development of the corpus callosum. Witelson and Nowakowski (1991) posited that asymmetrical axon growth may have been one of the factors that establish anatomic asymmetries or lateralization within an individual. The results from all three studies failed to support the hypothesis that any directional asymmetry is apparent in the growth of CC axons, because the amounts of growth axons in each hemisphere exhibited did not differ significantly between sides. Experiment 1 was subject to methodological difficulties. Therefore, this experiment did not allow an unequivocal conclusion that directional asymmetry is not present in contralateral CC growth. However, Experiment 2 focused on CC axon development in the ipsilateral cortex and conclusions from this study is clear: there is little evidence for

any directional asymmetry in ipsilateral axon growth prior to the CC reaching midplane.

The second hypothesis tested was whether or not an organismic growth gradient may be present in an individual to direct an asymmetry toward one side of the body (Brown & Wolpert, 1990). Initially, to determine if a gradient existed across systems, both the eyes and paws were also tested for asymmetry. Body weight was the common metric of this set of studies and as such, allowed comparisons between different developing systems. Figure 43 illustrates that no directional asymmetry is apparent in the eyes or paws. If an asymmetry gradient existed in mice it would be individual, because at the population level none of these systems exhibited a skewed distribution. To test more comprehensively for an organismic growth gradient, separate systems within an individual were investigated. The fetuses obtained for Experiment 2 were also used in Experiment 4. However, due to the damage incurred during perfusion only eleven subjects provided measurements for the paws in addition to all cortical areas. The results from these experiments do not support the contention that a common asymmetry gradient may exist in separate systems of an individual. Figure 44 shows the growth difference between sides, expressed as body weight, for each cortical region and paw measurement. All asymmetries were calculated as R-L, so any negative differences (to the left of the solid line) were indicative of left side growth advantage. It is apparent from this Figure that for the majority of subjects, no hemisphere showed a growth advantage across cortices. Furthermore, there appears to be little or no correlation between cortical and paw growth asymmetries. A closer look at Figure 43 shows that only one fetus (subject

Figure 43. A frequency distribution of R-L differences for estimated body weight, as derived from CC axon distance, eye height and widest interdigit angle using all subjects from each experiment. This figure shows that the degree of R-L differences was approximately equal between the brain, eye and paw; and that side differences showed normal distributions indicating that fluctuating, rather than directional, asymmetry was apparent.

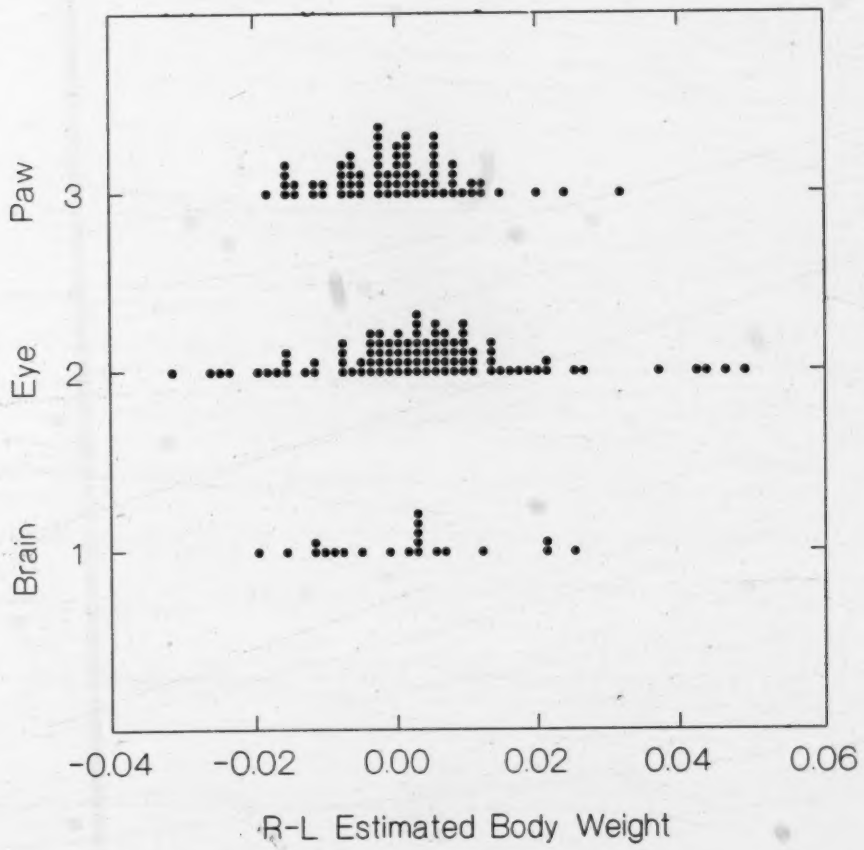
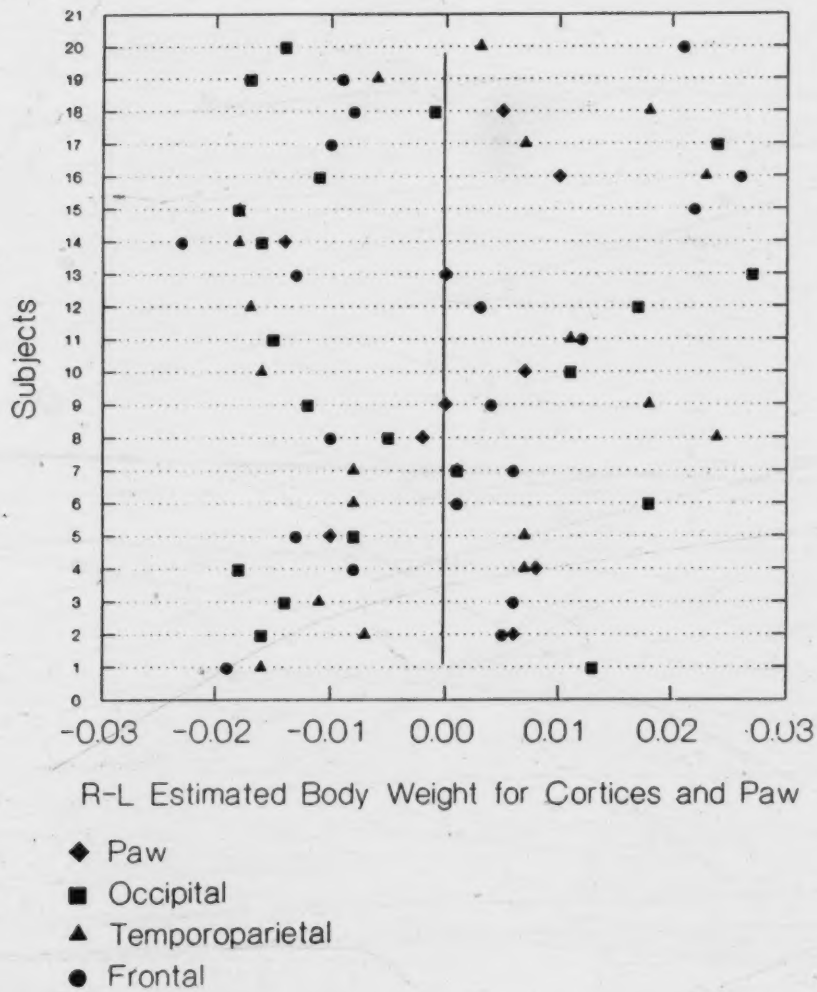


Figure 44. Scatterplot of the R-L differences in estimated body weight for both B6D2F$_2$ and BALB/cWah1 fetuses, calculated from axon growth in all three cortical areas and interdigit angle 3. This figure shows that the direction of an asymmetry does not appear to correlate highly between systems. Note: paw measurements were only available for eleven subjects.



14) demonstrated the pattern of growth characteristic of an organismic asymmetry gradient. In this fetus all four systems displayed a left side advantage. Palmer and Strobeck (1986) in reviewing the measurement and analysis of fluctuating asymmetry (FA) had reached similar conclusions. The authors pointed out that asymmetry within an individual rarely showed a consistent pattern and that a slight directional gradient in one system did not strongly predict the direction of a gradient in another system. Unfortunately, in the present set of experiments it was not possible to study eye closing in concert with the brain and paws of a single fetus. Stronger conclusions could have been drawn about asymmetrical growth gradients in an organism had all three systems been studied. However, to complete a study such as this would require modifications of current laboratory methods. As discussed in Experiment 4, alternate perfusion techniques would be required to fix brain tissue successfully without incurring the subsequent paw or eye damage.

The lack of directional asymmetry in the prenatal mouse may not be entirely unexpected when the behavioral patterns of mice are taken into account. Evidence of lateralization in the mouse has been primarily represented by the paw-preference work pioneered by Collins (1985). The work of Collins and others (Signore et al., 1991; Biddle et al., 1993; Waters & Denenberg, 1994) has consistently found that a skewed distribution of paw preference is present only in individuals, not across populations. The results from Experiments 1 and 2 are in agreement with the conclusions that within the mouse corpus callosum individual asymmetries are the norm rather than the exception. A possible interpretation of these data would be that the right-left differences in cortical

measures were indicative of an individual anatomic asymmetry reflecting an individual lateralization. In contrast, the asymmetry in CC growth may not be reflective of an underlying laterality, because results have also been obtained that have found little or no correlation between the size or shape of the CC and a behavioral measurement (e.g. Bishop, Kruyer & Wahlsten, 1996; Bulman-Fleming et al., 1992; Laroche & Ward, 1992). However, the present set of experiments was conducted in fetal mice, and because mice do not show strong population asymmetries, the generalizability of the results regarding CC growth and laterality to non-mice subjects may be limited.

The fluctuating asymmetry data gathered from each experiment were relevant to the investigation of the third hypothesis, that hybrid organisms will show a greater degree of symmetry compared to inbred organisms (Wahlsten & Wainwright, 1977). One reason that has been forwarded for the increased asymmetry of inbred organisms is that homozygotes are less stable (i.e. less fit) than heterozygotes (Soulé, 1979). It has been thought that homozygotes may be less fit than heterozygotes because the gene products of a homozygous organism are suited for a more narrow range of environmental conditions. In contrast, it has been suggested that the gene products of a heterozygote are more robust and better able to function over a wider spectrum of environmental conditions. However, the results from the present set of studies failed to support the contention that inbred organisms develop significantly more asymmetrically than their hybrid counterparts. In each experiment Strain was the between factor in the ANOVA used to test for directional asymmetry and only experiments 3 and 4 revealed a significant strain effect. Furthermore, the F-tests conducted on the fluctuating asymmetry

indices were only significant between strains in Experiment 3. There may be a number of reasons as to why the results from these experiments did not support significant strain differences in asymmetry.

It has been proposed that asymmetry is greater within systems not closely linked to the fitness of the organism (Soulé & Couzin-Roudy, 1982); however, the characteristics that were measured in these experiments may have not suitably reflected an organism's fitness. The evolutionary fitness of an organism is defined as that organism's level of adaptation to an environment such that the higher the level of fitness the more capable that organism is of reproducing and perpetuating its genes. Figure 43 illustrates that the spread of right-left differences is approximately equal between the brain, eyes and paws. Therefore, it may be inferred that either none of these systems was more closely linked to fitness than any other, or possibly that there is little relationship between the symmetry of brain, eye and paw development and evolutionary fitness.

Second, the strains used in these two experiments did not truly reflect a test of genetic homozygosity versus heterozygosity. To test this idea would require using two homogeneous populations, one homozygous and one heterozygous. This comparison could have been achieved if the inbred C57BL/6J and DBA/2J strains had been contrasted to their first generation offspring (i.e. B6D2F_1 fetuses). However, in the present study B6D2F_2 fetuses were used as the standard population. These fetuses were a second generation population and therefore according to Mendelian genetics would exhibit an equal number of homozygotes and heterozygotes, thereby making the population heterogeneous. This prevented an effective comparison of the effects of

genetic homozygosity versus heterozygosity between two populations because the heterozygous population in fact contained a number of individuals who were genetically homozygous.

Fluctuating asymmetry has traditionally been labelled as random deviations from perfect bilateral symmetry (Van Valen, 1962). For the subjects in each experiment the measurements were a "snapshot" of the extent of development in that individual, and in all of these experiments fluctuating asymmetry was evident. The finding of a side advantage in the brain that appears random is tentative support for the possibility that the hemisphere which has the growth advantage is flexible, and may shift from right to left and back again during the course of development. That is, if it were possible to cut and measure the same brain at two different times the side with the furthest extent of CC growth in the first measurement may not be the same during the second measurement. It would appear from the results obtained in the first two experiments that asymmetry of cortical growth is not pronounced while the CC is growing within the ipsilateral cortex. This conclusion does not preclude the possibility that asymmetrical CC growth may have a causal relationship to the development of behavioral or anatomic asymmetry; if this is the case these differences may be apparent in fetuses where the CC has already proceeded into the contralateral cortex. A study of this sort is feasible using the technique of double labelling. However, the limitations that arose during Experiments 1 and 2 should be taken into account if successful results are to be obtained. For instance, a mechanical delivery system should be used to ensure symmetrical dye application. But, rather than a pipette filled with a single type of dye, it would be optimal to develop a

delivery mechanism that can inject two different dyes simultaneously. Despite the methodological difficulties a study of contralateral CC development might provide useful information. For example, does a directional asymmetry exist beyond midplane, or does the amount of fluctuating asymmetry increase when CC axons grow contralaterally?

In summary, the set of experiments discussed above have failed to find support for the hypothesis of an organismic asymmetry gradient. Additionally, if asymmetrical CC growth does have a role in establishing lateralization or anatomic asymmetry within a population, it is not during ipsilateral axon growth. Using the common metric of body weight it has been established that asymmetry in the central nervous system is not less than in external morphology. Also, within the developing mouse fetus the amount of asymmetry appeared to be increased for inbred rather than hybrid strains. Unfortunately, the significance of this trend could not be elucidated within the present experiments because the inbred strains were compared to a heterogeneous population that obscured direct comparisons between homozygotes and heterozygotes. Finally, the results from the present set of experiments suggest that further research in the area of asymmetry concentrate on development of CC axons in the contralateral hemisphere and investigate a wider range of systems within an individual.

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